

CHITOSAN in the Preservation of Agricultural Commodities



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Chitosan in
**THE PRESERVATION
OF AGRICULTURAL
COMMODITIES**

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Chitosan in **THE PRESERVATION OF AGRICULTURAL COMMODITIES**

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PREFACE

In June 2014, the First International Meeting, with the title *Chitosan and Its Applications on the Preservation of Agricultural Commodities* was held in Morelos, México. This outstanding event brought together relevant scientists and students working with chitosan in the fields of agriculture from different countries including Cuba, Italy, South Africa, Venezuela, and México. This meeting made clear the necessity of summarizing and updating available information on this compound associated within agriculture areas, specifically in dealing with its application for the conservation of horticultural commodities.

Regarding this compound it is important to note that over the past decade, chitosan (the deacetylated form of chitin) polysaccharides have gained enormous importance in diverse scientific disciplines (e.g., pharmaceutical and biomedical areas), where literature has been systematically published. Furthermore, in these two disciplines, applications of chitosan now include among others, nutrition supplements, drug delivery, gene therapy, tissue engineering, and wound healing. Fortunately, advancements have shown positive results also in the area of agriculture. The presence of amino groups ($-\text{NH}_2$) in its chemical structure has resulted in chitosan exhibiting unique and ideal properties in different agricultural systems, including food conservation and food security through development of biodegradable edible coatings and films containing natural antimicrobials. Chitosan has also been integrated into programs of biological disease control due to its documented antimicrobial properties for controlling pathogenic microorganisms in various horticultural commodities. It has elicitor properties that enhance the natural defenses of fruits, vegetables, and grains. Chitosan is also being considered in microdevices to be integrated in “intelligent” and active packing for extending fruit and vegetable shelf life.

Chitosan can be extracted from diverse marine organisms, insects, and fungi. It has been considered a biodegradable and biocompatible material, not associated with toxicity or side effects. Presently, the use of chitosan has been technologically justified in sustainable agriculture programs because it raises no public health and safety concerns. In the fresh produce industry, this compound is safe for the consumer and the environment, and it has been approved by the US Food and Drug Administration (FDA) as a “generally recognized as safe” (GRAS) food additive. Likewise, regulation

EU 2014/563 included chitosan chloride as the first member of a basic substance list of plant protection products (planned with Regulation EU 2009/1107).

This specialized book on chitosan includes the following sections:

The first section, *Chitosan Obtention and New Materials-Based Chitosan*, explores the close relationship between the chemical characteristics of chitosan with its main functional properties, and the current functional chitosan derivatives. It explains the relationship between chitosan structure and its modifications and the specific properties in the final product as well as its potential of application, particularly in the agricultural and horticultural food sectors. The second section, *Biological Activity, and Mode of Action of Chitosan*, examines the effects of novel integrated applications of chitosan coatings, alone and in combination with other technologies such as modified atmosphere packaging, plant derivatives, plant gums, physical treatments, organic polymers, organic salts, and antagonist microorganisms in the preservation of fresh fruits, vegetables, and grains, including minimally processed products. The enzymatic and microscopic defense mechanisms against plant diseases and pests following chitosan application are also considered. The third section, *Biochemical and Molecular Aspects of Chitosan*, deals with the main mechanisms proposed to explain the beneficial effects observed when chitinous materials that are employed to control fungal diseases in plants. These effects include the occurrence of a fertilizer effect, indirect inhibition of the pathogens via their decomposition by-products, stimulating/supporting growth of profitable microorganisms, and elicitor activity of chitin. Likewise, studies of the gene expression during the chitosan–*Colletotrichum*–avocado interaction are presented. The fourth section, *Chitosan Bionanocomposites*, gives insight on the use of chitosan nanocomposites in biological models associated with fruit and vegetable conservation. Aspects related to the development of chitosan bionanocompounds, main nanoparticle obtaining methods, and environmental implications associated with the use of nanomaterials in the agricultural area were also considered.

The contributors to this book are nationally and internationally recognized scientists with wide experience in the study of different aspects of chitosan. Throughout recent years they have been active researchers in the field of chitosan, focusing in different aspects of agriculture and food conservation, which is demonstrated by their numerous high-impact publications on chitosan properties and applications. We editors acknowledge and thank their enthusiastic interest in participating in the writing of this book.

We are also deeply grateful for the continuous support and encouragement of this idea from the Elsevier team: Patricia Osborne (Acquisitions Editor), Jackie Truesdell (Editorial Project Manager), and Susan Li (Production Department).

The Editors

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PART 1

Chitosan Obtention and New Materials Based-Chitosan

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CHAPTER 1

Chemical Characteristics and Functional Properties of Chitosan

Jaime Lizardi-Mendoza, Waldo M. Argüelles Monal,
Francisco M. Goycoolea Valencia

INTRODUCTION

Chitin is an aminated polysaccharide biosynthesized in several invertebrate animal species. It is a main compound of the exoskeleton of arthropods, the most abundant animal phyla that include insects and crustaceans. The presence of chitin in some microorganisms such as bacteria, fungi, protozoan, and algae species has also been reported [1]. Chitin is considered one of the most abundant biopolymers and is regarded as one of the substances with highest production and degradation rate in the biosphere. Therefore, chitin plays an important role in the biogeochemical cycles of carbon and nitrogen, mainly in aquatic ecosystems [1,2].

Despite chitin being almost as abundant as cellulose and sharing several chemical and functional features with it, chitin applications are restricted to a few specialized fields. There are several reasons for this; one is that the natural structures of chitin that could be easily used are scarce (in contrast with cellulose structures such as wood or cotton). Other reasons are the difficulties in obtaining stable chitin solutions or the product variability associated with the inherent diversity of the sources and extraction procedures. Therefore, most of the chitin production is dedicated to obtain chitosan, which is a deacetylated derivative. After the deacetylation process, the resulting chitosan has diverse functional groups: some capable of being ionized, the amino moieties, and also the remaining acetamide groups that are prone to form hydrophobic associations. This chemical characteristic of chitosan has influence in many functional properties of this molecule (i.e., at acid pH values, the amino groups become cationic, promoting the dissolution of chitosan). Also the polycationic character of chitosan allows it to interact with diverse types of molecules. This, together with its structural capacities, biocompatibility, and other properties, make chitosan attractive for producing functional materials applicable in several fields.

CHITOSAN: DEFINITION AND SOURCES

Chitosan is the term used to denominate the polymer of glucosamine and *N*-acetyl glucosamine where the deacetylated units are present in major proportion or their distribution in the polymer chain is such that allows it to be dissolved in aqueous diluted acid solutions. A distinctive feature of the chemical structure of chitosan (Figure 1.1) is the predominant presence of units with amino groups that can be ionized. These groups become cationic in acidic media promoting the chitosan dissolution and polyelectrolyte behavior in solution.

In 1859, Rouget obtained an acid soluble fraction of chitin after boiling it in a concentrated potassium hydroxide solution [3]. This is considered the first scientific report of chitosan. Subsequently, the synthesis and occurrence of chitosan in diverse organisms, mainly fungi, was proved. However, its natural abundance is minimal compared with chitin abundance. Hence, most of the chitosan is produced by thermochemical deacetylation of chitin.

Multiple procedures have been proposed and developed to obtain chitosan and comprehensive reviews on the theme are available [3–5]. Most of the existing methods to produce chitosan are variations on thermo-alkaline deacetylation of chitin using hydroxides at high temperatures (e.g., $>80^{\circ}\text{C}$). The industrial production of chitosan today is based on this type of process. Chitosan could be also obtained by homogeneous chemical deacetylation of chitin [6]. Alternatively, biotechnological procedures have been proposed, including extraction from cultures of selected fungi strains or enzymatic deacetylation of chitin with limited success [7,8].

Typical chitin deacetylation by a thermo-alkaline procedure is a heterogeneous phase reaction, where the process conditions (e.g., starting material quality, particle size, reactants mixture ratio, additives, agitation rate, etc.) have a determinant influence on the characteristics of the produced chitosan. Consequently, notable variability on the functional properties of produced chitosan has been observed [9]. To achieve uniform production with proper quality control, an accurate monitoring through the entire process is required. Nowadays, consistent quality values and standardized

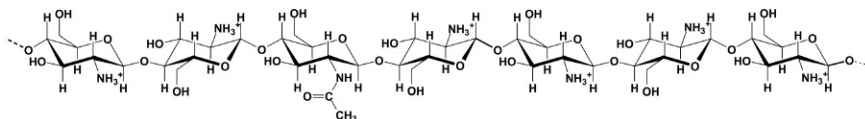


Figure 1.1 Chemical Structure of Chitosan. Schematic interpretation of the polycationic state of chitosan (acetylation = 1/7) in acid aqueous solutions.

methods to characterize chitosan intended for diverse applications are being discussed and adopted [9–11].

Homogeneous alkaline deacetylation of chitosan has been also reported [6,12]. Slow random deacetylation of the chitin molecules takes place in alkali chitin solutions at room temperature. This method of deacetylation could take up to 48 h to produce chitosan with DA of less than 10% in a single treatment. It was demonstrated that chitosan homogeneously obtained with DA around 50% is soluble in distilled water. The random distribution of the deacetylated units along the polymer chains generates a balance that allows chitosan to be dissolved in neutral water [6]. An alternative procedure using the chitin swelling in cold alkaline solution has been used to produce homogeneously deacetylated chitosan. In this procedure a mixture of chitin in concentrated NaOH (10–50% wt) goes through several cycles of freezing and thawing until a chitin solution is obtained or the chitin particles are swelled. Subsequently it is heated (up to 100°C) to proceed with the homogenous deacetylation [13,14].

Chitosan is a substantial component of the cell wall of certain fungi, particularly those belonging to the class Zygomycetes [15–17]. The enzymatic deacetylation of chitin has been proved as a synthesis mechanism of chitosan in fungi. This enzymatic modification plays a role in the regulation of the interactions of such fungi with plants [7,18,19]. Hence, the two main biotechnological alternatives to the chemical production of chitosan are the fermentation of chitosan containing fungal strains and the use of chitin deacetylases. Considerable research has been conducted in the production of chitosan by fermentation using different strains and suitable methods for the extraction of fungal chitosan [15,17,20–22]. Some advantages of this chitosan production method include obtaining chitosan free of allergenic animal proteins. The molecular weight and DA of fungal chitosan can be controlled by varying the fermentation conditions with no demineralization. The fermentative production of fungi on cheap biowaste is a continuous and unlimited source of chitosan. Additionally, concurrent extraction of β -glucans, another bioactive biopolymer, is an extra value-added product [5,8,15,23]. Despite these advantages, only few documented commercial ventures are based on biotechnological chitosan production.

PHYSICOCHEMICAL CHARACTERISTICS

The physicochemical characteristics of chitosan are polydispersed due to the variability associated with sources and production process conditions. To understand the functional properties of chitosan as a consequence of

measured characteristics such as polydispersity should be considered. This also emphasizes the need for precise and standardized methods to determine such characteristics, primarily as they apply to the degree of acetylation and the molecular weight as the main characteristics that influence most of the properties of chitosan. Other characteristics such as purity, crystallinity, inorganic matter content, and water content, among others, could be also relevant in the context of each intended chitosan application [10,11].

Crystalline Structure

In the solid state, chitosan molecules are generally organized in highly ordered crystallites contained in considerable amorphous regions. There are two main chitosan crystalline polymorphs [24,25]. One is called the “tendon chitosan” polymorph, which is a hydrated form and is the most common. The “annealed polymorph” is an anhydrous crystal form. In both polymorphs, the crystal cell is formed by two antiparallel chitosan molecules with a twofold helix conformation stabilized by hydrogen bonds. Differences among polymorphs arise from the presence of water molecules between crystal cells stabilizing the structure by multiple hydrogen bonding [26]. Through a heating treatment, it is possible to transform “tendon” form to the “annealed” form; this transformation is irreversible [26].

For chitosan salts with organic and inorganic acids, up to four crystalline polymorphs have been reported. Type “I” salts are mostly anhydrous; in these crystals the backbone chitosan chains retain the extended twofold helix of the unmodified chitosan molecule. This conformation of chitosan is called *type I form*. Conformational change of chitosan molecule occurred by salt formations of types II, IIa, and III, where the chains are arranged in antiparallel helicoids of differentiated fold [24–27].

Degree of Acetylation

The proportion of acetylated and deacetylated groups, or degree of acetylation (DA), determines the distinction among chitin and chitosan. Chitin deacetylation reaction progresses by exposing amino groups along the molecule. The extent and distribution of this modification causes several changes in the main properties of the molecule. One of the most notorious changes is that, as the amino groups are ionizable, chitosan becomes polycationic in acidic media. This unusual quality for a biopolymer allows chitosan to be capable of forming solutions and actively interacting with diverse molecules. Thus, the DA determines most of the properties of chitosan, including solubility, extent of swelling in water, susceptibility to

biodegradation, bioactivity, and biocompatibility among others. Practically, the DA has influence in all the functional properties of chitosan.

As a fundamental feature in relation to its properties, the accurate determination of the DA is required for characterization and quality control of produced chitosan. Several analytical tools have been used to measure DA, most of which depend on the dissolution of chitosan in aqueous acid solvents. Therefore, a single technique cannot typically be adopted to cover the full range of DA. The most-used techniques are based in potentiometry, UV spectroscopy, ^1H -NMR and infrared spectroscopy [3,27–30]. The existing methods have specific advantages and drawbacks. Additionally, DA values discrepancies are frequent in measurements of the same sample with different methods. Hence, the use of an absolute method such as NMR is recommended whenever possible; otherwise, it is necessary indicate precisely the method and general conditions used.

Absolute methods are those that do not require external standards and previous calibrations to determine the DA values accurately. The best example of this type of method is NMR. Some advantages of NMR are that it does not need gravimetric measurements; the purity of chitosan does not need to be known accurately as long as the impurity peaks do not overlap with the relevant peaks of chitosan; the peaks used for DA determination in this method are well resolved; and the integration of these peaks is straightforward. Also, DA can be calculated using different combinations of peaks in order to verify that the method is consistent. Due to these advantages, together with the precision that can be achieved, NMR techniques are usually employed as the standard to calibrate other methods [27,29,31,32]. High-resolution ^1H - and ^{13}C -NMR spectroscopy could be used to estimate DA of chitosan samples in solution. Solid-state ^{13}C - and ^{15}N -NMR spectroscopy could also be used to analyze chitosan samples without the need of dissolving, although specialized equipment and accessories would need to be implemented. Liquid phase ^1H -NMR is the most suitable method for the determination of the DA of chitosan. The technique has been proven fast, precise, reproducible, rugged, robust, and stable. Usually, the determination of the DA of chitosan by this method is performed in 2% DCl solutions at 70°C [27,29,31,32]. Recently, alternative ^1H -NMR operation settings have been proposed, achieving comparable results more rapidly by using milder conditions (room temperature) [27,31]. The use of partially deacetylated chitosan samples is recommended in order to improve the solubility and obtain high-quality spectra in solution state. It has been demonstrated that limited depolymerization by nitrous acid treatment does not significantly

Table 1.1 Absorbance band used to estimate degree of acetylation

Bond	Vibration type	Wavelength (cm ⁻¹)	Use
O—H	Stretching	3450	Reference
N—H	Stretching	3360	Reference
C—H	Stretching	2878	Reference
C—H	Deformation (pyranose ring)	1420	Reference
C—O—C	Stretching in glycosidic linkage	1150–1040	Reference
N—H	Bending in secondary amide (amide II)	1560	Probe
C=O	Stretching in secondary acetoamide (amide I)	1660	Probe
NH ₂	Bending in amino group	1590	Probe
C—N	Stretching in secondary amide (amide III)	1320	Probe
CH ₃	Rocking in acetoamide group	1380	Probe

modify the estimation of DA or dyad sequence distribution [31]. The DA could be calculated by several proposed relationships of signal integrals of the glucose ring and the acetyl group protons [32].

Infrared spectroscopy is one of the most widely used and studied methods for determining DA, because of its simplicity and availability. The basic methodology to calculate DA involves the measurement of a probe band and an internal reference band. Numerous relationships of probe and reference bands have been proposed to determine the DA of chitin and chitosan samples [28,33]. Table 1.1 includes a number of absorbance bands used to estimate DA. The quantity of methods proposed suggests that the FTIR DA determinations depend on sample form, treatment, and calculation procedure. Therefore, quantitative FTIR analysis should be performed meticulously, and calibration with respect to an absolute technique is recommended. Nevertheless, FTIR without calibration has been proved to be a functional tool to determine differences in DA and crystalline structure of chitosan [29].

Molecular Weight

The molecular weight of chitosan is a characteristic that has determinant influence in most of its functional properties [34–36]. Together with the degree of acetylation, it is the most important chemical characteristics of

chitin and chitosan. These characteristics have marked effect on the functional properties of chitosan either in solution or solid state. The molecular weight has particular influence on the viscoelastic properties of solutions and hydrated colloidal forms.

As many other natural polymers, the molecular characteristics of chitosan are polydisperse. This is particularly applicable regarding to the molecular weight. Chitin presents heterogeneous distribution of molecular weights as result of constant synthesis and degradation processes in living tissues and depolymerization during the extraction procedures. In chitosan, the molecular weight is also result of depolymerization that takes place through the deacetylation process. Consequently, a given sample is typically a mixture of chitosan molecules of assorted sizes. To describe the heterogeneous distribution of this fundamental property, several molecular weight averages are used. Namely, number – average molecular weight (M_n), determined by osmometry; viscosity – average molecular weight (M_v), estimated by viscosimetry; weight – average molecular weight (M_w), measured by light scattering (LS); and Z-average molecular weight, obtained by sedimentation analysis. In a typical distribution curve (Schultz–Flory distribution), the average values are related to each other as follows $M_n < M_v < M_w < M_z$ with an approximate proportion 1:2:3 for $M_n:M_w:M_z$. The polydispersity index (PDI) of a sample is defined as M_w divided by M_n and gives an indication just how wide the distribution could be [37].

Several analytical techniques are available to estimate the average molecular weight of the chitosan. Most of these chitosan characterization systems rely on high-efficiency separation techniques, such as size exclusion chromatography that allows the determination of molecular weight distribution [38]. The viscosimetry and dynamic light scattering techniques are commonly used to determine the molecular weight of chitosan in diluted solutions. It is recognized that most accurate information about size, behavior, and conformation of a polymer is obtained from solutions in dilute regime. However, many factors, experimental and relative to the intrinsic of the studied material, should be considered for a proper determination of the parameters that describe characteristics of the polymer. Regarding chitosan, experimental factors that have evident influence on analytical determinations include the characteristics of the solvent system (pH, ionic force, temperature, etc.), solution aging, as well as the purification and fractionation procedures, among others. All these factors, predominantly the solvent features, influence the solute–solvent interactions that determine the polymer conformation and hydrodynamic volume. The DA and units distribution

pattern are examples of intrinsic factors that should be controlled in analytical determinations. There are numerous reports about chitosan characterization in dilute solutions that have generated valuable conclusions; however, due to the quantity of possible combinations of factors, unresolved controversies remain [38–40].

The intrinsic viscosity, $[\eta]$, is a characteristic parameter of a polymer under specified solvent and physicochemical conditions (i.e., temperature, ionic strength, etc.). The $[\eta]$ is directly proportional to the average molecular weight of the polymer, accordingly to the Mark–Houwink–Sakurada (MHS) equation (Equation 1.1). Several sets of constants (k and exponent α) reported for chitosan correspond to differences in solvent, ionic strength, pH, temperature, type, and concentration of cosolvent, as well as molecular weight and degree of deacetylation of the chitosan used [38,41–44]. Therefore, it is advisable to report the used set of MHS constants in the molecular weight estimation of chitosan to avoid ambiguities.

$$[\eta] = k \cdot M_v^\alpha \quad (1.1)$$

Techniques of molecular separation such as size exclusion chromatography (SEC) have been used to study chitosan solutions corresponding to narrow molecular weight distributions [45–47]. Determination of the molecular weight and molecular weight distribution by SEC demands either proper molecular weight standards with a narrow molecular weight distribution or the use of appropriate detectors to determine absolute molecular weight (i.e., light scattering), viscosity, and concentration. Dextrans and pullulans, flexible and uncharged polysaccharides, have been used as standards for estimation of molecular weights of rigid polysaccharides such as chitosan, resulting in overestimated molecular weights. Better results have been obtained calibrating the SEC system with chitosan fractions with narrow molecular weight distribution obtained from preparative SEC. In some cases, the presence of aggregates could induce the overestimation of the M_w in light scattering measurements [48]. An adequate solvent system selection is required to achieve an accurate characterization of the polysaccharide in solution. The solvent system should reduce the ionic interactions with the stationary phase, avoiding adsorption, ionic inclusion and exclusion; similarly, it should stabilize the hydrodynamic volume of the polymer [47].

Given the influence of the molecular weight on the properties of chitosan, it should be available with a molecular weight range of 3–4 orders of magnitude in order to satisfy the requirements of its various applications [49]. Consequently, diverse efforts to manipulate the molecular weight of

chitosan have been undertaken in order to control or improve its properties (e.g., reduce viscosity or enhance water solubility). Typically, the molecular weight of chitosan is reduced by physical, chemical, or enzymatic hydrolysis. Physical means provide the added energy needed to break the glycosidic linkage; for this, thermal, sonic, irradiation, and hydromechanical methods have been used effectively to depolymerize chitosan [50–52]. Acid, alkaline, and oxidative reactions have been used to chemically hydrolyse chitosan [35,49,53]. Alternatively, the use of chitinases and other hydrolytic enzymes have also been applied to obtain low molecular weight chitosan or oligomers [54–56].

FUNCTIONAL PROPERTIES

The chemical characteristics assembled in chitosan provide it with distinctive properties. Chitosan is a linear copolymer of close related units; one is the deacetylated version of the other that displays dissimilar features. Acetylated units, *N*-acetyl glucosamine, could form hydrogen bonds and hydrophobic interactions that stabilize the molecule, providing it with certain rigidity and reinforcing its structural properties. On the other hand, the amino group of the deacetylated units, glucosamine, could be ionized ($pK_a \approx 6.5$) to turn chitosan into a cationic polyelectrolyte, an unusual property for a biopolymer. Moreover, chitosan amino and hydroxyl groups are susceptible to chemical modifications that could enhance its functionalities. These chemical characteristics allow chitosan to set diverse interactions with inorganic and organic compounds. The chitosan features are complemented by diverse biological properties that include biocompatibility, biodegradability to harmless products, almost an inexistent toxicity, and specific interactions with diverse living tissues (bioactivity).

Solubility and Solution Properties

Contrasting with chitin, chitosan can be dissolved in aqueous media [57,58]. Chitosan could be considered a weak base, because the amino groups distributed along the chitosan molecule could be protonated. It could get a charge density as high as one cationic charge per glucosamine unit. Thus, chitosan could go through typical neutralization reactions of alkaline compounds and dissolve in acid aqueous solutions [23,58]. Diluted inorganic acids, such as hydrochloric, phosphoric, carbonic, nitric and perchloric acids, are good solvents for chitosan. An exception is the sulfuric acid that generates the insoluble chitosan sulfate. Chitosan forms soluble salts in numerous

organic acids such as acetic, lactic, formic, as many other mono- and dicarboxylic acids. Normally, fatty acids are not suitable solvents for chitosan [12].

Most nonpolar organic solvents cannot dissolve chitosan. There are reports of limited chitosan solubility in dimethylformamide and dinitrogen tetroxide, in a 3:1 chitosan to solvent ratio. In these solvents chitosan forms low viscosity solutions without chemical modification of the polymer [59]. Another report describes dissolution of diverse chitosan salts (formed with *p*-toluenesulfonyl acid, camphorsulfonic acid, methanesulfonic acid, and salicylic acid) in dimethyl sulfoxide. To achieve the dissolution of chitosan in organic solvents is one important reason that drives the chemical modification of chitosan [60].

Chitosan thermally degrades before reaching glass transition, which is typical for polysaccharides with high hydrogen content. Therefore, preparation of any kind of materials such as films, gels, sponges, fibers, particles, and so on should be done through chitosan solutions. Furthermore, most of the functional properties of chitosan are exhibited or enhanced in solution state [36]. Chemical characteristics (i.e., DA, unit distribution, chain length, etc.) and solution parameters (i.e., ionic strength, pH, temperature, dielectric constant of the solvent, etc.) are both responsible for the chitosan molecules conformation in dilute solutions. The chitosan behavior under the influence of the aforementioned parameters has been extensively studied, concluding that, in diluted regime, chitosan tends to behave as a worm-like chain with considerable stiffness [13,36,43,61]. Similarly, the average polymer solubility parameter (δ_{pt}) has been estimated at $40.7 \text{ J}^{1/2}/\text{cm}^{3/2}$ for chitosan of 36% of DA in aqueous HCl solution [36].

As chitosan becomes a polycationic in acid solutions, the dissociation potential (pK) is an essential parameter to characterize the polyelectrolyte behavior of chitosan. The pK_0 , intrinsic pK , or pK value at theoretical zero charge of chitosan varies within 6.46 and 7.32, depending on DA [62]. It has been demonstrated that chitosan solubility requires a protonation degree (α) of 0.5 or higher [42,58,63]. Temperature, electric charge density and distribution, as well as screening of these charges by small ions affect the hydrodynamic volume and the chain flexibility of chitosan [58]. Intra- and intermolecular electrostatic repulsions due to protonation are enhanced at low pH, generating higher solubility, chain expansion, and therefore larger macromolecular hydrodynamic volumes. However, in the presence of large amounts of small anions, such as chloride, the viscosity decreases with pH due to electrostatic charge screening that enhances the chain flexibility. Three distinct domains of chitosan behavior in solution have been

identified and related to the DA: First, the polyelectrolyte domain for DA below 20%; then, a transition domain between $20\% < \text{DA}$ and $< 50\%$, where chitosan becomes less hydrophilic; and third, the hydrophobic domain for $\text{DA} > 50\%$, where polymer association forces prevail [43]. Static and dynamic light scattering as well as fluorescence studies indicate the tendency of chitosan to aggregate. This tendency has been related to the polysaccharide concentration, DA, and pH of the chitosan solution. It is thought that the molecular associations are due to the formation of both hydrophobic interactions and hydrogen bonding [13,38,43,64].

The rheological characteristics of chitosan solutions are key factors for many applications. Chitosan solutions behave generally as a typical non-Newtonian shear-thinning fluid [65,66]. Rheology studies indicate that the viscosity and flow properties of the solutions vary with the DA of chitosan. The viscosity, the non-Newtonian flow properties and the flow activation energy ($E\gamma$) tend to increase as the chitosan DA decreases. The presence of salts decreases the viscosity and the non-Newtonian flow properties, but they do not change the $E\gamma$ of the chitosan solutions. In solution the chitosan macromolecules have a tendency to entangle and form physical networks due to abundant intermolecular hydrogen bonding, even at low concentrations. The density of the molecular entanglements in the chitosan solution depends on concentration, temperature, and shear rate applied [66,67].

The dilution regimes can be related with the evolution of the hydrodynamic volume of the chains as the polymer concentration change. In a dilute regime, when the polymer concentration is lower than C^* (the overlap concentration), the macromolecular chains are isolated, with minimal influence over other polymer molecules in solution. Over the C^* concentration, at the semidilute regime, the entanglement processes arise. Finally, the concentrated regime is when the chain dimensions become independent of the polymer concentration [66]. The viscoelastic behavior of chitosan solutions fit in to a master curve, which describes the dependence of the specific viscosity η_{sp} on the entangling parameter $c[\eta]$ with a pending slope of 1.4 in the dilute regime, up to $c[\eta] \approx 4$. A slope of 1.16 up to a $c[\eta]$ of 1.45 has been reported for chitosan solutions in 0.3 M acetic acid – 0.2 M sodium acetate. This could indicate the boundary where incipient molecular contacts and entanglements begin [65,67,68].

Normally chitosan does not dissolve in water at neutral or alkaline pH. However, some reports claim that chitosan with DA about 50% and random deacetylated group distribution can be effectively be dissolved in water. This type of chitosan could be prepared by controlled homogeneous

deacetylation or by chemical N-acetylation of chitosan [6,69]. Alternatively, water-soluble low molecular weight chitosan could be obtained by extensive chemical or enzymatic depolymerization [56,70,71]. Water solubility is one of the main aims of chitosan chemical derivatization. Most chitosan salts are readily soluble in water, producing acid solutions.

Structural Capacity

A chitosan molecule retains most of the structural capacities of its precursor, chitin. Furthermore, the polycationic character of chitosan permits the formation of diverse complexes (e.g., ionic, electrostatic, etc.) and allows chemical derivatization to modify or enhance its material-forming capacities. Chitosan could form particles, capsules, films, fibers, gels, and porous structures by itself or in composites with other polymers, proteins, lipids, metals, and other organic and inorganic compounds.

Chitosan lacks thermoplastic properties and degrades before melting, and for those reasons, most of the procedures to generate structured materials use chitosan in solution. The diagram in Figure 1.2 includes the common mechanisms to obtain solid structures from aqueous acid chitosan solutions. Chitosan becomes insoluble, increasing the pH of the solution above its pK_a (Fig. 1.2A). Another way to obtain chitosan coacervates is by reducing the solvent capacity of the media without degrading it. This could be achieved mixing the solution with miscible nonsolvents (i.e., alcohols, acetone, etc.),

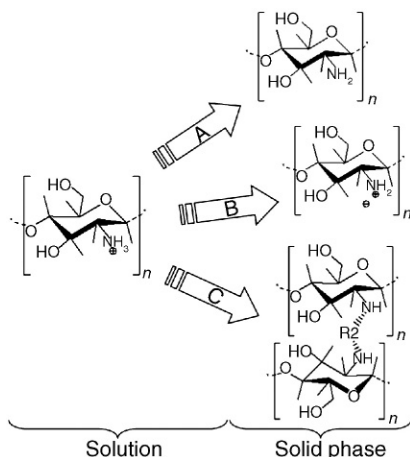


Figure 1.2 Formation of Solid Structures from Acid Chitosan Solutions ($pH < 6$). (A) Neutralization. (B) Water removal (drying) or addition of a miscible nonsolvent. (C) Chemical cross-linking. R1, anion; R2, multivalent anion, polyanion, or other covalent cross-linker.

adding enough quantity of nonanionic cosolute, or removing the water from the chitosan solution (Fig. 1.2B). In these cases, the solids obtained are comprised of chitosan salts formed with the acid used to dissolve it. Generally, those solid chitosan salts are water-soluble. Alternatively, chitosan solid structures can be produced that link several chitosan chains by chemical bonds; these bonds commonly are ionic or covalent (Fig. 1.2C). Multivalent anions, anionic polyelectrolytes, and molecules with at least two functional groups (i.e., dialdehydes) are used to form bridges between chitosan chains [72–74]. The properties of the obtained materials will depend on the characteristics of the chitosan used (i.e., DA, molecular weight), type and extent of their interactions with added compounds, and the process conditions (i.e., solution properties, cosolvent, casting method, etc.). Typically, chitosan solutions of concentration below C^* produce particulates or materials with poor mechanical properties. Above C^* , topological contacts and molecular entanglements occur providing connection points to develop extensive linkages, which is the base of the mechanical capacities of materials such as films, fibers, or gels.

The solid structures of chitosan could be also divided into two classes, namely chemical and physical, according to the type of cohesive forces involved. This classification was proposed by Berger and collaborators for chitosan hydrogels. Chemical structures are those formed by irreversible covalent links, while physical structures are those formed by various reversible links that could be ionic, hydrophobic, or other weak interactions. In chemical structures, chitosan molecules are interconnected by cross-linkers, which are molecules with a well-defined molecular weight and smaller than the connected chains. This class includes structures of chitosan cross-linked with itself, hybrid polymer networks, and interpenetrating polymer networks (IPN). In the first case, cross-linking involves two structural units that may or may not belong to the same chitosan polymeric chain. Hybrid polymer networks are those where cross-linking reaction occurs between a chitosan unit and a unit of a polymeric chain distinct of chitosan. In IPNs, chitosan and other polymer are mixed together but the cross-linking reaction only bond units of the same type. Semi-IPN is that where just one polymer is cross-linked. When both polymers are cross-linked, the two entangled cross-linked networks formed are denominated full-IPN. The physical structures include ionic cross-linking, polyelectrolyte complexes, and structures formed by physical associations (e.g., hydrophobic interactions, hydrogen bonding, etc.) of chitosan or its derivatives. Ionic cross-link structures are similar to those with covalent cross-links. The electrostatic

attraction between the cationic amino groups of chitosan and the anionic groups of the other polyelectrolyte is the main interaction leading to the formation of the polyelectrolyte complexes [72–74].

Numerous procedures have been reported to obtain chitosan-containing structures intended for diverse applications. Chitosan micro- and nanoparticles, beads, and capsules have been produced by ionic gelation, sieving, emulsion, coacervation–precipitation, and reverse micellar methods. The main use of these chitosan particles are in drug delivery systems [75–81]. Cast evaporation, spraying, and layer-by-layer techniques have been used for film formation using chitosan or its polyelectrolyte complexes [82–86]. Several spinning variations (i.e., wet-spinning or electrospinning) are used to obtain chitosan fibers up to the nanoscale; diverse dope mixtures and coagulation systems have been reported. Similar procedures could be applied to produce nonwoven chitosan fabrics [87–91].

Gel is a semi-solid state formed by a macromolecular network swollen in water or other fluids. Chitosan gel formation methods correspond to the previously described classification [73]. Several techniques of each type of chitosan gels have been proposed and used in diverse applications. Dialdehydes such as glutaraldehyde and glyoxal are commonly used to produce chemical cross-linked chitosan gels; although several other molecules with at least two functional groups have been used as covalent cross-linkers [74,92–94]. Preparation of several types of interpenetrating networks of chitosan have been reported, most of them with synthetic polymers [95–97]. Ionic cross-linked gels of chitosan could be obtained with metallic anions, as Mo(VI) or Pt(II), or with polyanionic molecules, such as triphosphosphate [77,98–100]. The interaction of chitosan with polyanionic polymers result in the generation of polyelectrolyte complexes. The most commonly used polyanions are polysaccharides bearing carboxylic groups, such as alginate, pectin, or xanthan [101–103]. Also, the use of proteins (i.e., collagen), synthetic polymers, and other macromolecules, such as DNA, have been investigated [104–106]. Additionally, several procedures to obtain chitosan gels formed by physical associative forces have been described, as the thermal gelation of chitosan glycerophosphate mixtures and the chitosan gelation in hydroalcoholic media [107–109].

Adsorption Capacity

Adsorption is a process involving ionic, hydrophobic, hydrogen bonding, or Van der Waals forces. Chitosan has functional groups that could generate these interactions with molecules of several types. Adsorption of proteins,

lipids, metals, and other substances, such as aromatic compounds and dyes, over chitosan have been studied. The adsorption capacity of chitosan is dependent on its physicochemical characteristics, mainly DA and molecular weight, the conditions of interaction (e.g., pH, ionic strength, etc.), and type and characteristics of the adsorbate [110,111].

The interactions of chitosan with proteins are mainly influenced by the pH that determines the degree of ionization of both compounds. To generate electrostatic interactions the isoelectric point of the protein should be lower than the pK_a of chitosan. Generally, chitosan–protein interactions are not strong, thus, proteins can be desorbed on appropriate conditions [110]. Protein adsorption on chitosan is displayed in chitosan functional properties as mucoadhesion or cell and microbial flocculation. Currently, the adsorption capacity of chitosan is fundamental on its main commercial uses, such as flocculating agent in wastewater or food-related applications [112,113].

Despite probings of the lipid adsorption capacity of chitosan, the interaction mechanisms are not completely elucidated [114,115]. Some authors indicate that cationic nature of chitosan leads to a strong interaction with lipids having an opposite charge. On the other hand, the formation of hydrophobic aggregates has been indicated as a cause of lipid–chitosan associations, regardless of the DA [114–116]. The lipid adsorption capacity of chitosan is related to a cholesterol-lowering effect when is consumed by humans and animals [117].

Chitosan is recognized as effective metal adsorbent [22,110,118]. Metal adsorption on chitosan implies ionic and chelating interactions; however, there is no universal agreement about the mechanisms for these processes. The adsorption capacity of chitosan and stability of the metal–chitosan complex varies mainly with the DA. It has been indicated that binding of metal ions increases with pH, indicating that only the nonprotonated amino groups can bind the ions [119]. Metal adsorption may be also influenced by mixing (mechanical or ultrasound) and the physical state of the chitosan (powder, gel, fiber, or film). The exceptional binding capacity of chitosan, greater than 1 mmol/g for most metals, is comparable to commercial ion exchange resins [118]. The affinity of chitosan for cations could be represented by the sequence: $\text{Cu(II)} \geq \text{Hg(II)} > \text{Zn(II)} > \text{Cd(II)} > \text{Ni(II)} > \text{Co(II)}, \text{Ca(II)}$ [119]. Most studies of metal adsorption of chitosan tend to determine whether chitosan will complex with a given ion, in order to determine the amount of ions that can be bound or to understand the involved processes [22,110,118–120]. However, comparison between different studies is complicated due to the large variability of experimental conditions.

Diverse types of dyes (except for cationic dyes) and phenolic compounds could also be adsorbed by and form complexes with chitosan. Dye adsorption appears to be driven by electrostatic interactions, being the DA of chitosan and the pH the main factors affecting the process. On the other hand, the association of chitosan with phenolic compounds seems to be formed and stabilized by hydrogen bonds [110,111,121,122].

Reactivity

In numerous reports on chemical modification of chitosan, the most common purposes for modifying chitosan include improvement of its solubility at neutral or alkaline pH, provision of its solubility in organic solvents, and improvement of specific functional properties. Chitosan normally could undergo the same derivatization reactions as chitin. However, the accessible amino groups of chitosan open the possibilities to other type of modifications. Typically, chitosan derivatization is made through chemical procedures. However, the enzymatic approach to modification of chitosan is interesting owing to its specificity and relatively lower environmental impact [123].

The hydroxyl and amino groups present in chitosan are main groups susceptible to chemical modification. The amino group is more labile to derivatization, thus they require protection if derivatization on hydroxyl groups is intended. Chitosan phthalate is obtained by reaction of chitosan with phthalate anhydride. This is a derivative soluble in organic solvents and a common way to protect the amino groups when derivatization of hydroxyl groups is intended. The phthaloyl group could be removed by treatment with hydrazine to regenerate the amino groups of chitosan [12].

The amino group is susceptible to acylation, Schiff base formation, alkylation, copolymerization, and generation of quaternary amino salts, among others. N-acetylation is the most common N-acylation of chitosan. This reaction is used to generate homogenous half-acetylated chitosan soluble in water. Schiff base formation is fundamental for several chitosan derivatizations. Free amino groups of chitosan could react with aldehydes and ketones to give the corresponding Schiff base. The imine group formed is stable in neutral or alkaline environments and can be reduced to produce N-alkyl derivatives [12]. Quaternarization of amino groups could be achieved by treatment of chitosan with methyl iodide in alkaline media. The N-methylene phosphonic chitosan is an anionic derivative that is synthesized reacting chitosan with phosphorous acid and formaldehyde at 70°C by 7 h [124]. Various methods for graft copolymerizations on

chitosan have been reported, but it is typically performed using cerium ions, 2,2'-azobisisobutyronitrile, γ radiation or redox systems to induce radical formation necessary to initiate the graft polymerization [12]. Vinyl monomers such as acrylonitrile, methyl acrylate, or vinyl acetate have been copolymerized onto chitosan. Nonvinyl monomers such as lactic acid, aniline, ethylene oxide, poly(ethylene glycol), monomers and oligomers of sugars and amino acids, as well as dendrimers are some of the molecules that have been successfully grafted on chitosan [12,81,110,124,125].

Biodegradability and Biocompatibility

Biodegradable materials have numerous applications in medical, agriculture, drug release, packaging, and other fields [126]. Biodegradation comprises natural processes involving organisms or living tissues by which diverse compounds are degraded, mineralized, and redistributed through elemental cycles, such as the carbon or nitrogen cycles. In these processes, microorganisms play a central role, therefore biodegradation can only occur within the biosphere [127].

Chitinases, chitobias, and other enzymes capable of degrading chitosan are ubiquitous in nature and have been detected in a wide variety of sources from prokaryotes to higher animals, including plants and yeasts [54,128]. The enzymatic degradation of chitosan generally leads to the release of amino sugars, which can be incorporated into glycosaminoglycan and glycoprotein metabolic pathways, or excreted as nontoxic compounds [123,129]. Other enzymes such as lysozyme and several proteinases could also degrade chitosan under proper conditions of temperature, ionic strength, and pH. As general rule for chitinases and nonspecific enzymes, the reaction rates increase with the degree of acetylation [130,131]. Chitosanases are enzymes that specifically hydrolyze chitosan; however, distribution is significantly reduced compared to chitinases. Chitosanases, which are completely absent in mammals, could be founded mainly in soil living Actinomycetes (especially the *Streptomyces* species) and in bacteria (*Bacillus* sp., *Vibrio* sp., etc.) [131].

Biocompatibility could be defined as the ability of a compound or material to be in contact with a living system without producing an adverse effect [132]. Chitosan is normally regarded as biocompatible; its reported toxicity LD₅₀ is about 16 g/kg (oral test in mice), which is similar to salt or sugar toxicity [128,130]. Results of several studies on the biological compatibilities of different chitosan preparations varied considerably depending on the physicochemical characteristics of the used chitosan (mainly DA

and molecular weight) and the experimental conditions (e.g., biological milieu, etc.) [75,128]. However, chitosan is generally considered safe for plants and animals.

Cytocompatibility of chitosan has been proved on numerous studies using diverse cell types including, keratinocytes, fibroblasts, osteoblasts, chondrocytes, neurons, hepatocytes, endothelial, vascular, and cancerous cells [133–137]. It has been reported that the DA of chitosan films does not affect the compatibility towards keratinocytes and fibroblasts. Furthermore, it is suggested that DA would also have no effect *in vivo*, or towards other cell types. However, the chitosan DA has determinant influence on cell adhesion and proliferation; as higher is the DA lower is the cell adhesion [138]. The chitosan adherence to cells in hard and soft tissues together with its biocompatibility are the base of several biomedical applications, as cell scaffolding, drug carriers and tissue engineering.

Bioactivity

Chitosan has raised considerable interest for its diverse bioactive effects. The polycationic nature of chitosan is the main base for diverse interactions with proteins, lipids, genetic material, anionic molecules, and other biological compounds. A broad spectrum of biological activities of chitosan has been reported, including its ability to induce resistance mechanisms and growth in plants; antiviral, antifungal, and antibacterial effects; and diverse effects of biomedical interest (e.g., hypocholesterolemic effect, genetic material transfection, wound healing promoter, etc.) [124,129,139–142]. The main bioactive properties of chitosan are briefly described here.

Chitosan is a structural component of the cell wall of several plant fungal pathogens. As part of its resistance mechanism, most plants have proteins, lectins, and enzymes capable of recognizing and degrading chitin and chitosan. Studies found that chitosan triggers the elicitation of phytoalexins without affecting the plant cell membrane permeability [17,19,125,143–145]. For this reason, chitosan and derived oligosaccharides have been used to induce or enhance the production of this type of compounds and other interest metabolites (e.g., isoflavonoids, taxol, etc.) [19]. Apparently, the antimicrobial potential and elicitor activity of chitosan combine to produce improved plant growth effects [125,131]. These effects and the physiological mechanisms involved are discussed in other chapters of this book.

Antimicrobial activity of chitosan has been recognized in several bacteria, fungi, and viral infections [125,146]. The extent of its effect is strongly influenced by the physicochemical characteristics of chitosan. Apparently,

the inhibitory effect of chitosan increases with the molecular weight. The antibacterial activity of chitosan is also affected by pH, with greater activity being found at the lower pH values. Similarly, this bioactivity of chitosan and its derivatives is also connected with several phenomena, including biodegradation, induction of natural resistance, membrane effects, or direct action against microbes [125,131,146,147]. Chitosan antimicrobial activity has been reported against diverse pathogens as well as food spoilage, including both Gram-negative and Gram-positive bacteria. Apparently, Gram-positive bacteria are more susceptible to chitosan action. Some results, such as those of selective inhibition or reports on the absence of antibacterial action of homogenous water-soluble chitosan by itself but high effect achieved in mixtures with amylose, make evident the need of deeper knowledge on mode of action of chitosan against different type of microorganisms [148,149]. About antiviral action, it is thought that chitosan causes no effect on virus growth but inhibits the infection process, presumably by stopping replication. However, it is still unknown which stage of replication is the target of chitosan [150]. Due to the relevance of this theme on agricultural applications, it is also more extensively discussed in other chapters of this book.

Several chitosan effects on higher animals have been reported. Again, the physicochemical characteristics of chitosan together with the nature and conditions of the interaction have significant influence on the observed effects. Numerous actual and potential applications of chitosan are based on its compatibility and physiological effects on animals and humans. Consequently, these aspects of chitosan are studied in detail and several reviews on related themes are available [129,134,141,151–158].

Ingestion and nutritional aspects of chitosan have been investigated for several animals and humans. The dietary effect of chitosan-supplemented feeds of farm and laboratory animals, including chickens, rabbits, rats, mice, and fish, has been reported [159–161]. Most of the reports coincide in normal or enhanced growth pattern and reduction of dietary fat absorption. Some authors have indicated possible long-term adverse effects on chitosan-supplemented diets due to lack of appetite and changes on digestive microflora [162].

Chitosan is considered a dietary fiber because it is not absorbed in the human digestive tract even when it could be partially degraded. The cationic character of chitosan favors the fat binding in the acid environment of the stomach, resulting in a reduction of lipid absorption [130]. Prebiotic capacity of chitosan oligomers has been also reported [163,164]. Several countries

have officially approved the use of chitosan as a food additive. In some Asiatic countries, including Japan, chitosan has been traditionally used as functional ingredient of health-promoting foods, including dietary cookies, potato chips, noodles, and vinegar products [128,165]. The fat-binding capacity and hypocholesterolemic effect of chitosan have been confirmed; though, it has led to an increase in nutraceutical market products containing chitosan that claim body fat reduction and weight loss [153,165–167]. Chitosan appears to be well tolerated in clinical trials; however evidence indicates that the effect of chitosan on body weight is minimal and unlikely to be of clinical significance [117,154]. Similarly, a comprehensive study of the metabolic effects of long-term dietary supplement use of chitosan in humans has been recommended. Possible adverse effects of chitosan in low dose and long-term ingestion include the disruption of digestive tract microflora and interference in micronutrients absorption, particularly liposoluble vitamins and minerals [166,167].

Generally, chitosan-based implant materials induce minimal to normal foreign body reaction [130]. The formation of normal granulation tissue, often with accelerated angiogenesis appears to be the typical course of healing, and major fibrous encapsulation occurs rarely. Acute edematous response with a significant accumulation of neutrophils close to the implants is often seen in the short term [158]. This response tends to dissipate rapidly and chronic inflammation does not develop. It has been suggested that stimulatory effects of chitosan on immune cells may play a role in inducing local cell proliferation and ultimately integration of the implanted material with the host tissue [168]. Enzymatic degradation of chitosan is dependent on the DA, thus highly deacetylated chitosan may last up to several months in vivo [128]. On the other hand, materials made of chitosan and its derivatives are capable of generating several physiological effects such as hemostasis, macrophage activation, anti-inflammatory as well as hyaluronan synthesis, angiogenesis, and granulation stimulation [153,169]. Most of these chitosan effects act synergistically to obtain complex results, such as wound healing promotion [141,156]. Based on these properties several applications have been developed, such as wound dressings, sutures, and diverse implantable devices for medical and veterinary use [152].

CONCLUDING REMARKS

The distinctive chemical characteristics of chitosan are the base of a set of remarkable functional properties that have raised considerable attention through the last decades. The structural properties, biological activity, and

Table 1.2 Functional properties of chitosan fundamental for diverse applications

Field	Chitosan functional features	Applications
Agricultural	Plant stimulant, antimicrobial, structural capacity, biocompatibility	Enhanced plant growth, pest biocontrol, feed additives
Food	Antimicrobial, structural capacity, biocompatibility, adsorption	Food additives, food packaging, nutraceuticals
Environmental engineering	Adsorption, structural capacity, reactivity	Metal removal/recovery, waste-water treatment, enzyme immobilization

reactivity of chitosan keep open the possibility for a variety of new applications and great potential use in diverse fields. Chitosan is one of the few cationic natural polymers, and it is certainly the most abundant. A significant proportion of the research on this polysaccharide investigates the application of the chitosan properties in numerous and diverse areas. Chitosan has properties that make it particularly suitable for applications related to agricultural, food, and environmental engineering (Table 1.2).

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CHAPTER 2

New Bioactive Biomaterials Based on Chitosan

Elsa Bosquez-Molina, Leonor Zavaleta-Mejía

INTRODUCTION

The current knowledge concerning the physical, chemical, and biological properties of chitosan recognizes it as a unique and versatile biopolymer with a wide range of applications as biomaterial. Further, the biocompatibility, nontoxicity, nonallergenic, film-forming, and antimicrobial activity features of chitosan also make it attractive for potential use as a bioactive material [1,2].

It is well known that chitosan is insoluble under neutral and basic pH solutions because of a strong hydrogen-bonding network structure. Among the different approaches that have been developed to improve chitosan solubility are its partial hydrolysis to obtain low molecular weight chitosan and chitoooligosaccharides (COS), which are considered as chitosan with a degree of polymerization (DP) less than 20 and M_w up to 10 kDa that are soluble in water [3–5]. These COS are relatively novel compounds with different biological functions for medical uses and a wide scope of applications to preserve foodstuffs as food additives or in the developing of biocides to control plant diseases. The complex nature of chitosan was used to obtain a chitosan- Zn^{2+} complex derivative with a higher antibacterial activity against *Escherichia coli*, compared to native chitosan [6]. On the other hand, because chitosan is a polyelectrolyte, that is, a positively charged polysaccharide (pH < 6) it has been combined to form polyelectrolyte complexes by the interactions with macromolecules carrying ionized/ionizable groups of opposite charge. These intermacromolecular complexes are highly practical as flocculants, membranes, skin substitutes, coatings, and binders in industrial applications, hence, they can be used as materials for controlled delivery of fertilizers, pesticides, herbicides, and insecticides in soil, reducing environmental damage caused by excessive abuse of agrochemicals [6,7]. Moreover, recent research has been oriented to enhance the functional properties of chitosan through blending or cross-linking with other natural

or synthetic polymers; both are convenient and effective in improving its physical properties for practical applications [8–10]. In this regard, it has been reported the enhanced antimicrobial activity by blending chitosan with different natural compounds such as garlic oil, carvacrol, vanillin, and grape seed extract as well as potassium sorbate and nisin [1,11,12].

One of the more recent research lines involves the grafting, that is, the addition of specific functional groups or polymer chains (copolymer in the resulting product) onto the chitosan backbone, leading to various derivatives with new or improved properties [13–20].

It is important to point out that the agriculture as well as the packaging and food sectors are under constant pressure to develop or adapt and apply appropriate new technologies to reduce chemical pollution and satisfy several demands such as a high quality and safety, and protecting foods with a biodegradable and environmentally friendly package. In this context and with the vision of sustainability, the opportunity to increase the use of bio-derived polymers is playing a significant role, although still on a small scale, in the development of agronomic biodegradable films and biocomposites and those related to packaging materials, paper, edible films, and coatings. Thus, the increasingly growing demand for biodegradable materials has created a very dynamic field.

All in all, chitosan and its derivatives have demonstrated great potential as biological material for a wide variety of fields and industrial applications. In the food industry, chitosan and their derivatives, with their inherent antimicrobial activity and film-forming ability, are used to extend the storage life through controlling the spoilage by microbial attack. The following sections highlight some of the recent studies on molecular modifications of chitosan with potential for application as a package or coating material to preserve the safety, nutritional, functional, and sensory quality of agricultural commodities.

PHYSICAL AND CHEMICAL CHARACTERISTICS OF CHITOSAN

Chitosan is a derivative from chitin, which is found naturally in the shells of crustaceans, the exoskeletons of insects, and the cell walls of fungi [21–23].

Chemically, chitosan is the simplest linear cationic amino polysaccharide composed of β -(1–4) linked D-glucosamine and N-acetyl-D-glucosamine with various compositions of these two monomers. According to the amount of N-glucosamine or N-acetyl-D-glucosamine into the molecule, it is named chitosan or chitin, usually the chitosan contains more than 50% of N-glucosamine and the physical properties of chitosan depend on the deacetylation degree.

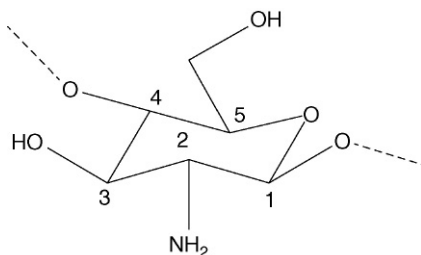


Figure 2.1 *N*-Glucosamine.

The higher deacetylation degree (DD), the higher free amino groups and a more positive charge in solution, which makes chitosan highly soluble in dilute organic acids such as acetic acid, lactic acid, and formic acid.

The amount of amino groups is also responsible of the different chitosan's structures and physicochemical properties, which in turn, are associated to their biological, chelation, and flocculation functions. Thus, several of its properties such as chelating metal ions, as well as the polyoxysalt formation, and ability to form films among others are attributable to the amino group [24]. The antimicrobial character of chitosan is due to its positively charged amino group, which interacts with negatively charged microbial cell membranes, leading to the leakage of proteinaceous and other intracellular constituents of the microorganisms [25].

The amino groups of chitosan are deprotonated at $\text{pH} > 7$ and the unshared electron pair readily react with electrophilic reagents such as aldehydes, acid chlorides, acid anhydrides, and epoxides [26].

The chitosan polymer can also be hydrolyzed because of the presence of glycoside bond in the molecule. This hydrolysis is called chitonolysis and produces chitosans with low molecular weight (M_w). Chitosans with low DP are called chitosan oligomers, chitoooligomers, or chitoooligosaccharides (COS), which are reported as having multifunctional properties [4,27,28].

It is important to emphasize that the amino groups at C-2 position as well as the two hydroxyl groups at C-3 and C-6 positions in chitosan are the functional reactive groups' key for chemical modifications (shown in Fig. 2.1) [10,21].

PRODUCTION OF CHITOOLIGOSACCHARIDES (COS)

As was mentioned before, the chitoooligosaccharides were among the first derivatives obtained by hydrolysis of chitosan. Unlike chitosan, its hydrolyzed products and COS are readily soluble in water due to their shorter

chain lengths and free amino groups in D-glucosamine units [29]. In this respect, the aim to develop an efficient process for reducing the chitosan M_w became of great interest to manufacture tailor-made chitosans of varying DDs and DPs [4,28]. To achieve this goal, several methods have been used including the action of reagents, oxidative-reductive depolymerization, enzymes, high-energy impact, or combination thereof. Because chemical hydrolysis has some drawbacks due to development of some toxic compounds and lower production yields, the enzymatic processes are now generally carried out in batch reactors and are both recommended and preferable as one of the promising methods to produce safe and bioactive COS. Detailed reaction mechanisms of the different ways of chitosan degradation as well as the current methods for their characterization are reported in excellent reviews [3,4].

In the latest research, many studies have reported several remarkable biological activities attributable to chitooligosaccharides, such as hypocholesterolemic [30–33], immunostimulating, antitumor, and anticancer effects [34–36], as well as accelerating calcium and iron absorption [33], anti-inflammatory, antioxidant, and angiotensin-I-converting enzyme (ACE) inhibitory activities, among others, all of them correlated with their structures and physicochemical properties [4,37].

The main feature reported for COS is their antimicrobial effect, thus, regarding the agricultural commodities field, their application is relevant because COS (DP2–8) have shown higher inhibitory activities against *Fusarium oxysporum*, *Phomopsis fukushi*, and *Alternaria alternata* than high-molecular-weight chitosan. It was also found that COS with higher degrees of polymerization (DP) have stronger inhibition of bacteria and fungus than chitosan alone and COS with lower DP [38–41]. Studies related to the COS structures and their antimicrobial effects show a correlation with the amount of protonated amino groups and relative molecular weights [23,42,43]. Regarding the elicitor activity, it has been reported that higher chitosan oligomers are effective elicitors and highly dependent on their polymerization and the presence of *N*-acetyl glucosamine [23]. Hence, in addition to the direct antifungal effect, COS has been shown to be effective in increasing activity of defense-related enzymes such as catalase (CAT), phenylalanine ammonia-lyase (PAL), and peroxidase (POD) [44] and to up-regulate gene expression of β -1,3-glucanase and chitinase [44,45] in the same way as chitosan. Moreover, the antioxidant response has also been associated closely with fruit resistance against disease, and these findings have been consistent in several studies carried out to maintain quality and

control disease during the postharvest life of some fruits such as grape berry (*Vitis vinifera* L.), strawberry (*Fragaria x ananassa*), raspberry (*Rubus idaeus*) [46], tomato (*Lycopersicon esculentum* Mill.) [47], and peach fruit (*Prunus persica* L.) [44].

Chitosan oligomers (COS) are easily soluble in water and, in this condition, exert superior antimicrobial, antitumor, and immune modulatory activities [48,49]. The preharvest application of these polymers has also proved to be remarkable in reducing the postharvest losses. Preharvest COS treatments inhibited various postharvest diseases of table grape (*V. vinifera* L.) [50], sweet cherry (*Prunus avium* L.) [51], and jujube fruit (*Zizyphus jujuba* Mill. cv. Dongzao) [52].

Moreover, COS and its derivatives can form physical barriers around the penetration sites of phytopathogens, preventing conidia from spreading to healthy tissues, germ tube elongation, and mycelial growth [53,54], such as *Botrytis cinerea* [55], *Penicillium expansum* [56], *Penicillium digitatum*, *Penicillium italicum* [57], *Rhizopus stolonifer* [58,59], and *Colletotrichum gloeosporioides* [60].

FUNCTIONALIZED CHITOSAN

It is well-known that the functional groups are specific groups of atoms within molecules that are responsible for the characteristic chemical reactions and properties of that molecule; so, in order to provide chitosan with a specific function, it can be functionalized by the addition of small moieties, oligomers, and even other polymers (grafting copolymers) onto the chitosan backbone (Fig. 2.2). The techniques for polymer functionalization and grafting involve processes that are closely related. One of the functionalizing-grafting techniques called “grafting-from” is the synthesis *in situ* of grafts of homopolymers or heteropolymers (small or long chains) containing functional groups. When the process involves the use of a preexisting polymer-possessing functional species and chain ends with chemical affinity to the backbone of the polymer (such as chitosan), the technique is named “grafting-onto.” Another technique of functionalization grafting is

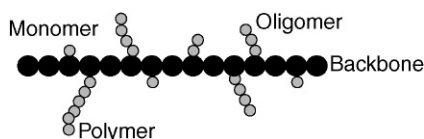


Figure 2.2 Schematic Representation of Graft Polymer.

carried out through the reaction or interchange of labile atoms of the polymeric backbone by functional monomers or reactive species. The development and application of graft polymers is a continuously growing scientific and technological field and has become a powerful tool to generate novel materials [61]. When branched chains are formed off a main chain of one type of polymer, one or several branches of a second type of polymer are called *graft copolymers*; the common nomenclature used for these copolymers is polyA-grafted-polyB or simply polyA-g-polyB [62].

Hence, a wide range of chitosan-based materials have been proposed by means of suitable chemical or enzymatic modifications onto the chitosan backbone to improve its solubility in water, antimicrobial properties, flocculant capacity, absorbency, and adhesiveness, while remaining biodegradable and biocompatible for potential applications in biomedicine, food, or water treatment [9,18,20,47,63–65]. Thus, the grafting with specific molecules makes evident the importance and the potential of the functionalization of chitosan to provide polymers with specific properties and desirable applications [66].

Throughout the past decade, the research has focused to a great extent on using the chitosan as a bioactive matrix, and the main chemical modifications made to this molecule have led to many derivatives as described in the following sections.

Chemical Modification of Chitosan

Grafting

The grafting method onto chitosan backbone is a more recent way to obtain chitosan derivatives with new or improved functionalities. It mainly consists of the insertion of small functional groups such as alkyl, carboxymethyl, saccharides, or oligosaccharides for increasing the chitosan solubility at neutral and alkaline pH without affecting its cationic character [9,64]. Other compounds (e.g., cyclodextrins) and acids (e.g., *p*-aminobenzoic, lactobionic, sialic, and polyacrylic) have also been attached in order to confer removal capacity of textile dyes, or drug delivery, wound healing, inhibition of microorganisms, antioxidant capacity, and increasing hydrophilicity [18,47,63,64,67].

Several graftings have been made to improve chitosan aqueous solubility. A carboxymethyl chitosan-grafted methacrylic acid (MAA) and hydroxypropyl chitosan-grafted MAA were prepared using ammonium persulfate (APS) as initiator in aqueous solution. These derivatives exhibited much better water solubility [65,68].

Another chemical modification to overcome chitosan's limited solubility was through the synthesis of carbohydrate-branched chitosan derivatives, using different carbohydrates such as L-fucose, D-fucose, N-acetyl-D-glucosamine, D-mannose, and β -D-lactose. It was found that the water solubility of the carbohydrate-branched chitosan derivatives depended on the degree of substitution (DS) values. The chitosan derivatives with less than 0.15 DS were insoluble, whereas those with more than 0.2 were soluble in neutral water (pH 7). The solubility in the latter case was greater than 10 mg/mL [69]. Water solubility and antioxidant capacity at neutral or basic pH of chitosan was obtained by specific attachment of fructose to amino function [70].

Graft of copolymers is synthesized to improve physicochemical properties of synthetic/natural polymers for applications in agriculture, biomedicine, and other fields. Different studies have been published on the grafting copolymerization of chitosan with various vinyl monomers such as acrylonitrile [17], methyl methacrylate, acrylamide [71], and acrylic acid, using cerium ammonium nitrate as redox initiator (by definition an initiator is any chemical species that reacts with a monomer to form an intermediate compound that is capable of linking successively with other monomers into a polymeric compound). Most initiators produce free radicals (e.g., peroxides and aliphatic azo compounds) that are usually employed to polymerize vinyl chloride, methyl methacrylate and other monomers.

Another interesting compound reported was a chitosan chemically modified by grafting polyacrylamide using ceric ammonium nitrate as the initiator and in the presence of *N,N*-methylene-bis-acrylamide as a cross-linking agent. These grafted products improved considerably the antibacterial activity against *Pseudomonas aeruginosa*, *E. coli*, *Bacillus subtilis*, and *Staphylococcus aureus* [17]. When acrylic acid or methyl-acrylate (MA) was grafted onto chitosan using the same initiator, the copolymer had higher thermal stability and degradability than native chitosan. A concentration of 50 μ L of acrylic acid–chitosan derivative revealed a higher antifungal activity against *Candida albicans*, *C. kefyr*, and *Aspergillus niger* whereas intermediate activity was exhibited against *Candida tropicalis*, *Candida krusei*, *Aspergillus flavus*, and *Aspergillus fumigatus*. On the other hand, the MA grafted onto chitosan showed an increasing antibacterial activity as the degree of grafting increased from 82–145% for *P. aeruginosa*, *E. coli*, *B. subtilis*, and *S. aureus* [17,71].

Chitosan-grafted acrylic acid for treating wastewater was also carried out, and it was found that the copolymer is a promising matrix for the

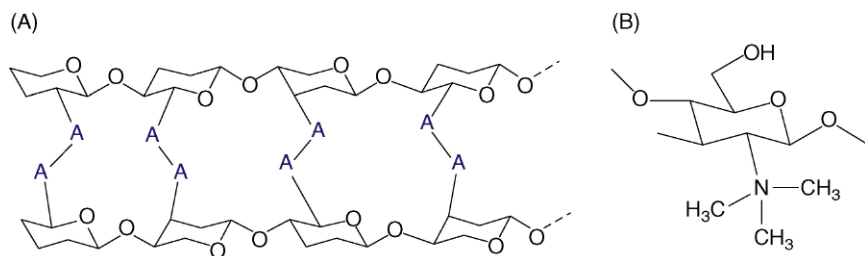


Figure 2.3 (A) *Chitosan Cross-linking* and (B) *Chitosan Quaternization*.

adsorption of metal ions such as Cr^{6+} and Cu^{2+} from metal solution. Hence, the chitosan-g-acrylic acid can also be used for wastewater treatment [72].

Chitosan has also been chemically synthesized by *N*-acylation with palmitoyl chloride for the purposes of achieving a higher hydrophobicity for ensuring a controlled release plus improved stability and adhesion to a fruit product. In this work, limonene (LIM) and peppermint (PM) were also incorporated into the modified chitosan to create bioactive edible coatings, which were tested for their ability to extend the shelf life of fresh strawberries during storage. Formulations based on modified chitosan containing LIM and Tween[®]80 perform better than other formulations. Therefore, the functionalized chitosan-based edible coating could become a promising method to carry specific antifungal agents without detrimental effects on strawberries and other fruits [73].

In other report, chitosan-based nanoparticles with a high degree of size uniformity were prepared by grafting D, L-lactic acid onto chitosan as a drug carrier. Hence, nanoparticles of ~ 10 nm in diameter were developed via a coprecipitation process by lactic acid grafted onto chitosan in ammonium hydroxide to form coacervate drops that increase drug loading and prolonged drug release. The lactic acid-grafted chitosan nanoparticles demonstrated a drug encapsulation efficiency of 96% and a protein release rate of 15% over 4 weeks [74].

Other chemical modifications made to chitosan include cross-linking and quaternization techniques. The cross-linking involves polymerization and postpolymerization stages that produce polymers with branched or cross-linked structures as illustrated in Figure 2.3.

The amine of chitosan allows for cross-linking of the polymer through a variety of cross-linkers such as diisocyanates, epichlorohydrin, *N,N*-disuccinimidyl suberate, and genipin, among others.

Chitosan cross-linked with the aglycone geniposidic acid (aGSA) resulted in a promising material as edible film with slower degradation rate,

relatively lower water vapor permeability, and retained antimicrobial ability as compared to the glutaraldehyde-cross-linked film [35]. Electrospun nanofibers were obtained using vapor-phase glutaraldehyde to cross-link chitosan; these materials have potential uses in a variety of industries such as medicine, packaging, agriculture, and automotive with specific applications in air filtration, protective clothing, substitutes for agricultural pesticides, and nanocomposites [75].

In order to improve the antimicrobial activity and physicochemical properties, chitosan was chemically modified to produce quaternary ammonium salts. Quaternization of *N*-alkyl chitosan derivatives was carried out using alkyl iodide to produce non-pH-dependent water-soluble cationic polyelectrolytes (*N,N,N*-trimethylchitosan, TMC). Additionally, the antibacterial activity was improved and TMC bioactivity was found to last longer [19].

One other interesting compound is the water-soluble chitosan derivative, *N*-[(2-hydroxy-3-trimethylammonium) propyl] chitosan chloride (HTCC), which was prepared by introducing quaternary ammonium salt groups on the amino groups of chitosan. The NMA-HTCC showed an excellent antimicrobial activity against both *S. aureus* and *E. coli* compared to chitosan alone, which does not show any antimicrobial activity at pH 7.2 [76].

Enzymatic Modification of Chitosan

Chitosan modifications by enzymatic methods offer alternative routes, especially in products to be applied in foodstuffs due to minimized hazards associated with toxic reagents, in addition to mild and environmentally friendly reaction conditions [9,12,18–20,77–79]. With respect to health and safety, enzymes eliminate the need for reactive reagents. Moreover, enzyme specificity may offer the potential for precisely modifying macromolecular structure for better control of polymer function [80].

In general, enzyme-catalyzed reactions are carried out in aqueous solutions where the hydrophilic residues of the enzyme are oriented on the surface of the molecule in contact with water for optimal catalytic activity; meanwhile the hydrophobic residues are buried in the interior of the enzyme molecule [81,82]. On the other hand, a variety of enzymes have been reported to be catalytically active with increased stability in the presence of organic solvents with small amounts of water offering benefits such as increased solubility of substrates in solvents with more efficient catalysis, less undesirable side reactions, and enhanced thermal stability of the enzymes,

among others. These changes in enzyme-catalyzed reactions in nonaqueous solvent systems have been exploited for the polymerization of phenols, selective interesterification of lipids, and synthesis of homopolymers and copolymers [83–86].

Studies about the enzymatic modification of chitosan started during the last decade of twentieth century and basically, two general approaches have been studied [12,85,87–89]. The first approach involves hydrolytic enzymes (e.g., lipases or proteases) under nonaqueous conditions to catalyze condensation or acyl-transfer reactions; however, a general limitation of this approach is that the reaction mechanism implicates the formation of a covalently bound, acyl-enzyme intermediate, which undergoes nucleophilic attack at the enzyme's active site [80,90]. Being that both the acyl intermediate and the polymeric substrate must bind to the active site, enzymatic condensation/transesterification reactions are susceptible to significant steric limitations and these reactions are typically slow. The second approach to polymer modification consists of employing an enzyme to generate a freely diffusible, reactive intermediate, which then undergoes reaction with the polymer. Because the polymeric substrate does not bind at the enzyme's active site, this approach is less susceptible to steric limitations.

Therefore, in the latter approach, the enzymes are used as active starting material in the reaction to produce freely diffusible reactive intermediates, so among the enzymes used with this purpose are the polyphenol oxidases (PPOs) such as tyrosinases, peroxidases, and laccases that convert phenols derivatives into *o*-quinones, which are electrophilic and can react with nucleophilic function, such as the amino groups of chitosan. Even though many reactions between *o*-quinones with nucleophilic groups have been extensively studied, quinone chemistry and the reaction mechanisms are not clearly understood yet [20,24,91]. By using oxidative enzymes, chitosan has been grafted with phenol derivatives to confer higher hydrophobicity and viscosity or new functionalities, such as the ability to adsorb cationic dyes, increased hydrophobicity, higher solubility in both water and organic media, and improved antimicrobial properties as well with new properties, such as antioxidant capacity.

It was reported that synthesis of chitosan–flavonoids conjugates by enzymatic treatment with chloroperoxidase (CPO, EC 1.11.1.10) increased the elongation and antimicrobial properties as well as the thermal degradability of the new chitosan derivative; it was also observed that the chitosan–quercetin films visibly decreased enzymatic oxidation when applied to cladodes (*Opuntia ficus-indica*) [66].

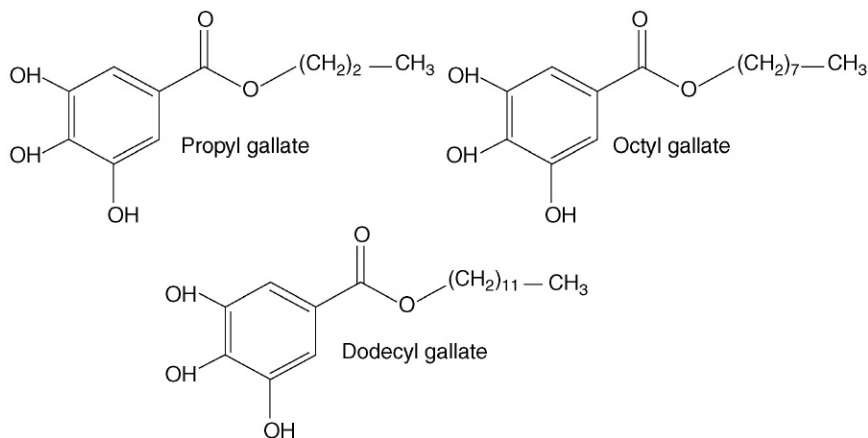


Figure 2.4 Gallates Esters.

The tyrosinase has been employed to graft hexyloxyphenol, chlorogenic acid, *p*-cresol, and flavonoids among others onto chitosan to improve the solubility, enhance the ability of the chitosan to form high-viscous solutions that can be converted into biodegradable gels [24,88,91–93]. In another report, tyrosinase was used for grafting the dipeptide Tyr-Ala and a glucosamine from casein hydrolysate onto chitosan. The grafted peptides conferred a higher viscoelasticity to solutions, a property that is important for a wide variety of foodstuffs and cosmetic products [94].

Another enzyme that has been used to functionalize chitosan is the lacase from *Myceliophthora thermophyla*. Chitosan has also been functionalized with ferulic acid (FA) and ethyl ferulate (EF); the resulting derivatives exhibited enhanced antioxidant properties, particularly the FA-chitosan derivative when compared with native chitosan. Both derivatives could be a model for applications in important technological domains such as active food-packaging and antioxidant additives for foodstuffs as well as for cosmetics [95].

Among the phenolic compounds grafted onto chitosan are the gallates, which are synthesized by the esterification of gallic acid. Propyl, octyl, and dodecyl gallates are used as antioxidant additives in foods (Fig. 2.4). Octyl gallate (OG) has shown strong antifungal activity against *Saccharomyces cerevisiae*, *Zygosaccharomyces bailii*, *C. albicans*, and *A. niger* [96]. It is important to point out that the main activity of OG is due to its ability to act as a non-ionic surfactant without need to enter the cell. Tyrosinase-catalyzed grafting of food-grade OG and dodecyl gallate to amino groups of chitosan and the resulting compounds were applied as solutions on plasma-activated biaxially

oriented polypropylene films and paper substrate. The final coatings exhibited strong antimicrobial activity against *S. aureus* and *Listeria innocua* as well as improved adhesion and hydrophobicity, making them potential functional additives for food-packaging materials [16].

The enzymatic grafting of phenolic compounds to chitosan was also achieved using phenol oxidase or peroxidase [20,24,88,91,97,98]. Recently, it successfully obtained chitosan-co-octyl gallate using horseradish peroxidase (HRP) as catalyst [20,81,82]. This enzyme has several advantages over tyrosinase, including a broader substrate range and an optimum pH for activity between 5 and 6, which is convenient for chitosan modifications because it is solubilized at pH below 6 [20]. The reaction involves two steps. In the first step, the hydroxyl radicals generated by the interaction between the redox pair of the components attack H-atoms in R-methylene (CH_2) or hydroxyl groups (OH) of the hydroxymethylene group of the chitosan. In the second step, HRP is oxidized by hydrogen peroxide to an enzymatic intermediate (Fig. 2.5). The intermediate state of the enzyme accepts a molecule of aromatic compound into its active site and carries out its oxidation resulting in a free radical, which is released to the solution leaving the enzyme in a state called E_{II} . This oxidized form of the enzyme molecule oxidizes to another aromatic compound, releasing a second free radical bulk solution and returning to the native form enzyme. These free radicals spontaneously combine to form a polymer [78,99].

The insertion of octyl gallate to the chitosan added value to the polymer, providing it with antioxidant capacity and increased antimicrobial activity without losing its filmogenic property (Fig. 2.6).

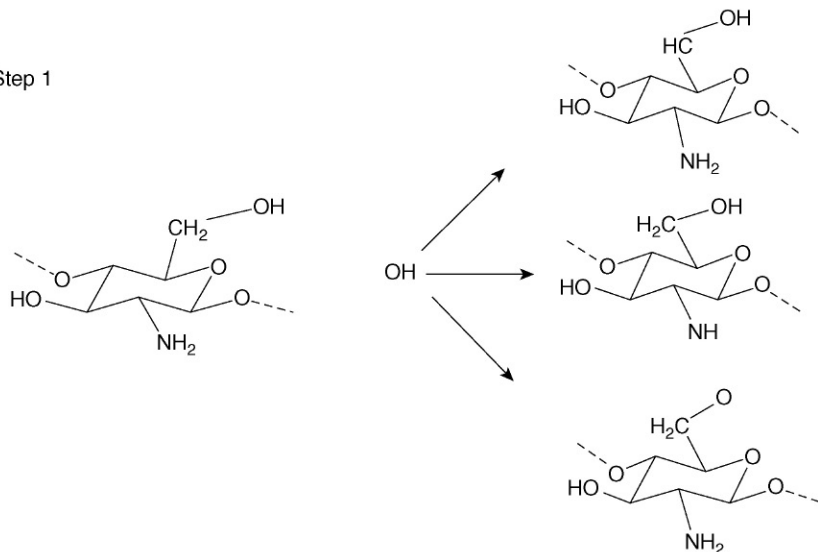
This copolymer showed an EC_{50} of 964 g/kg of DPPH as antioxidant power, as well as good viscoelastic properties, adhesiveness, and antimicrobial activity. The chitosan-g-octyl gallate solutions in acetic acid own remarkable viscoelastic features that provide structural and mechanical stability, which are desirable properties for the manufacturing of biodegradable materials such as edible films or coatings, scaffolding cells, drug delivery material, and moisturizer among others with potential application in food preservation, biomedicine, or cosmetics.

Some selected chitosan derivatives are presented in Table 2.1.

NANOCOMPOSITES

As it is now well known, nanotechnology is a discipline that involves the manipulation or self-assembly of individual atoms, molecules, or molecular clusters into structures to create materials or devices with new and different

Step 1



Step 2

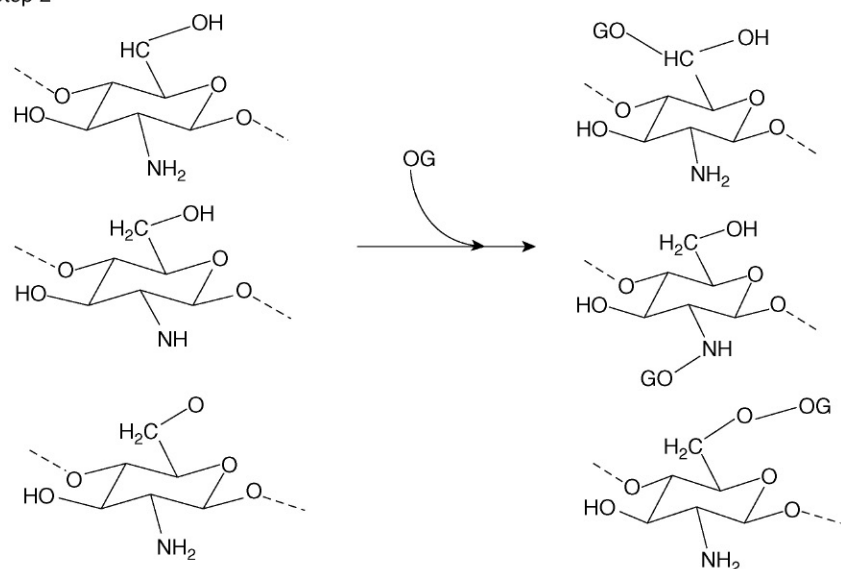


Figure 2.5 Proposal Mechanism of Octyl Gallate Grafting onto Chitosan.

properties. The word *nanotechnology* is generally used when referring to materials with the size of 0.1–100 nm, and as result of their size, these materials exhibit different properties from micrometric and larger materials in terms of physical strength, chemical reactivity, electrical conductance, magnetism, and optical effects [109].

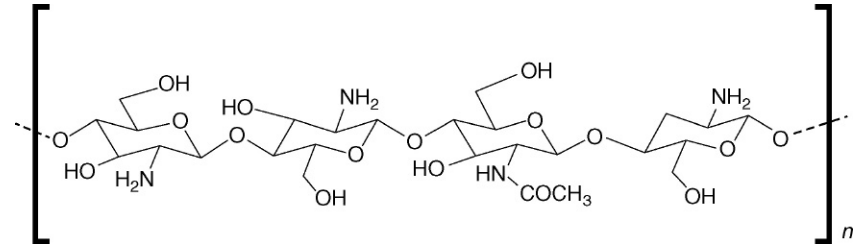


Figure 2.6 Chitosan-g-octyl Gallate.

Table 2.1 Chitosan derivatives and their uses

Chitosan derivative	Modification	Uses
N-alkyl chitosan	Chemical modification using alkyl iodide	Effective antimicrobial biopackagings [19]
Chitosan gallic acid	Conjugation using carbodiimide	Antioxidant polysaccharide for food preservation, cosmetics, and biomedical products [100]
Chitosan quercetin bioconjugates	Conjugates by enzymatic treatment with chloroperoxidase	Quercetin-chitosan with improved antioxidant and antimicrobial properties and retention of thermal degradability; diminish browning of <i>O. ficus indica</i> cladodes [66]
Chitosan chlorogenic acid	Enzymatically modify chitosan	Soluble under both acidic and basic conditions, and has a pH-region near neutrality in which it is insoluble [91]
Chitosan grafting gallates	Enzymatic grafting	Functional additives in food-packaging materials with modified surface hydrophobicity or improved adhesion toward other biopolymers [16]
Glutaraldehyde cross-linked chitosan microspheres	Cross-linking	Drug carrier for controlled delivered drug [101]
Ferulic acid chitosan or ethyl ferulate (ferulic acid ethyl ester) chitosan	Enzymatic grafting of the laccase	These new polymers can be a model for applications in important biotechnological domains, such as active films and antioxidant additives [102]

Table 2.1 Chitosan derivatives and their uses (*cont.*)

Chitosan derivative	Modification	Uses
Aglycone geniposidic acid cross-linked chitosan	Cross-linking	The aGSA-cross-linked increased its ultimate tensile strength, had a lower cytotoxicity, a slower degradation rate, and relatively lower water vapor permeability [35]
Polypropylene carboxymethyl chitosan	Irradiated with corona discharge	Antifungal activity against <i>F. oxysporum</i> , <i>V. albo-atrum</i> , <i>R. solani</i> , and <i>A. alternata</i> ; controlling postharvest fruit rots during storage and delaying the onset of infection [103]
Chitosan/poly(vinyl alcohol)/pectin ternary film	Blending	Antimicrobial activity of the film against pathogenic bacteria, <i>E. coli</i> , <i>S. aureus</i> , <i>B. subtilis</i> , <i>Pseudomonas</i> , and <i>C. albicans</i> [104]
Chitosan films with tannic acid	Cross-linking	More rigid structure, increase of tensile strength and the decrease of elongation as well as water vapor permeability [105]
Wheat gliadin/chitosan blend film	Blending	Blend films exhibit water vapor permeability and moisture absorption values; are expected to serve as antimicrobial packaging and coating for foods [106]
Coating based on gum arabic and chitosan	Composite	Can be used commercially for extending the storage life of banana fruits for up to 33 days, stored ($13^{\circ}\text{C} \pm 1$ and $80 \pm 3\%$ RH) for 28 days, and then for 5 days at simulated marketing conditions (25°C and 60% RH) [107]
Kudzu starch-chitosan		The composite film using malic acid as solvent showed antimicrobial activity against <i>E. coli</i> and <i>S. aureus</i> [108]
Chitosan-caffeic acid derivatives	Grafting with 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride	The maximum IC_{50} of radical-scavenging activity (0.064 mg/mL) was observed at the highest caffeic acid-containing derivative and these derivatives were water-soluble [98]

Nanosized materials are currently considered new materials and have recently received relevant attention because of their potential in areas such as health care, textile, agricultural, food, and packaging industries.

In the context of the agricultural industry, nanotechnology offers new tools for the molecular treatment and control of diseases, enhancing the ability of plants to absorb nutrients, using nanostructured catalysts that allow lower doses of pesticides and herbicides in maintaining or increasing their efficacy; it is worth mentioning that nanotechnology can also be used to clean ground water. Meantime, the impact of nanotechnology research in the food and packaging industries has been clearly visible because it encompasses specific problems from the production, processing, and packaging up to the enhancing of food quality through selected additives or improvements to the way the body digests and absorbs food [109].

The novel nanocomposite packaging materials with antimicrobial functions have a high potential for an active food packaging; these systems are particularly effective because of the high surface-to-volume ratio and enhanced surface reactivity of the nanosized antimicrobial agents, making them able to inactivate microorganisms more effectively than their micro- or macro-scale counterparts. Commonly used or tested antimicrobial nano and nanocomposite materials also include natural biopolymers such as chitosan, or natural antimicrobials (thymol, carvacrol, isothiocyanate, nisin, antibiotics), as well as enzymes, among others.

Then, bionanocomposites are hybrid organic-inorganic materials, which are extraordinarily versatile in that they could be formed from a large variety of biopolymers and also from different inorganic solid particles. Some of nanocomposite materials with chitosan include the chitosan/layered silicate nanocomposites, which are of interest for advanced biomedical materials in tissue engineering, artificial bones, or gene therapy. Other possible fields of applications are related to their mechanical, thermal, and barrier properties, making this class of materials attractive for potential uses in controlled drug and pesticides delivery, membranes for food processing, drinking water purification, oxygen barrier films, and food package. In this respect, it was found that chitosan/vermiculite nanocomposites had enhanced thermal stability [110]. PEG-grafted chitosan nanoparticles were developed as delivery material of colon-targeted peptide drugs; through these microspheres, the drugs are protected against peptide digestive enzymes and the low pH in both the stomach and small intestine [101].

Other chitosan–montmorillonite nanocomposites were prepared by an ion exchange reaction between water-soluble oligomeric chitosan and a Na^+ –montmorillonite. The chitosan–montmorillonite nanocomposites were rapidly prepared due to the high affinity between the chitosan and the montmorillonite clay host. The thermal stability of chitosan was remarkably improved in the interlayer space due to the strong electrostatic interaction of cationic chitosan molecules with anionic silicate layers. These nanocomposites showed a synergistic effect in the antimicrobial activity against *E. coli* and *S. aureus* [111].

Recently, novel nanocomposite films (thickness 0.13–0.2 mm) of chitosan, starch, glycerin, cyclophosphamide, and 2–10% Fe_3O_4 nanoparticles were prepared for different applications either in the food industry or the medical field. The film containing 10% Fe_3O_4 nanoparticles showed the greatest Young modulus, and the stability of the nanocomposites increased by addition of Fe_3O_4 nanoparticles. The *in vitro* antibacterial activity effects for the films were greater against *S. aureus* [112].

Other types of chitosan-based nanocomposite films were also prepared using a solvent-casting method to incorporate four types of nanoparticles using an unmodified montmorillonite (Na-MMT), an organically modified montmorillonite (Cloisite 30B), a nano-silver, and an Ag-zeolite (Ag-Ion). Mechanical and barrier properties of chitosan nanocomposite films were affected through intercalation of nanoparticles. Thus, the tensile strength increased by 7–16%, whereas water vapor permeability decreased by 25–30%, depending on the nanoparticle material tested being higher with Na-MMT and the lowest with Cloisite 30B. In addition, the silver-containing ones showed a promising range of antimicrobial activity against *S. aureus*, *Leuconostoc monocytogenes*, *Salmonella typhimurium*, and *E. coli* [113].

Chitosan/layered silicate nanocomposites with different ratios were successfully prepared. The nanocomposites showed antibacterial effect on Gram-positive bacteria. The lowest minimum inhibition concentration (MIC) value of the nanocomposites against *S. aureus* and *B. subtilis* was 0.00313% (w/v) [114].

In order to improve water barrier properties, blends of chitosan (from Cuban lobster) and clay micro/nanoparticles, which were prepared by dispersion of the clay in the film matrix, were studied. The water vapor barrier properties of the films were significantly improved by incorporation of clay in their composition, while the water solubility decreased as the clay concentration increased (for a constant chitosan concentration). The tensile strength of chitosan/clay films increased significantly with increasing

chitosan and clay concentrations, while the values of elongation decreased slightly with increased chitosan concentration [115].

Recently, nanocomposite films from two chitosan acetate solutions with different amounts of chitosan were prepared, with Na-MMT and/or glycerol. The Na-MMT enhanced the stiffness (up to 100%) and strength (up to 70%) and a dramatic decrease in elongation at break (up to 75%) was noted. Both water sorption and water vapor permeability decreased due to the presence of dispersed Na-MMT nanoparticles [116].

A new bionanocomposite film with high potential for application on food packaging was made with chitosan, nano zinc oxide, and neem essential oil. In this material, the nano zinc oxide incorporated improved the tensile strength, elongation, film thickness, film transparency, and decreased water solubility, swelling, and barrier properties due to the presence of neem oil and nano zinc oxide in the polymer matrix. It also showed a good antibacterial activity against *E. coli*. [117].

APPLICATIONS OF CHITOSAN DERIVATIVES IN AGRICULTURAL COMMODITIES AND PACKAGING INDUSTRY

The diverse range of applications for chitosan in agricultural commodities has been directed primarily to the development of films and coatings because the chitosan-based films have a high potential to prolong shelf life and control senescence and decay of many fresh fruits, such as peach (*P. persica* L.), strawberry, lychee (*Litchi sinensis* Sonn.), and grape [46,118,119] as well as for minimally processed products such as sliced mango (*Mangifera indica* L.), apple (*Malus domestica* Borkh.), and papaya (*Carica papaya* L.) to retain color, original taste, and texture [33,120,121].

Later studies used combinations of chitosan with other biopolymers or incorporating natural antimicrobial agents into chitosan formulations for edible films mainly due to the improved properties of blend films compared to those prepared only with chitosan. Some of this type has enhanced antimicrobial efficacy or antioxidant properties. The antimicrobial activity has been tested against food pathogenic bacteria, namely *E. coli*, *S. aureus*, *S. typhimurium*, *L. monocytogenes*, and *B. cereus* [1].

Formulations based on blends of biopolymers and chitosan were designed to obtain a matrix for the controlled release of antioxidants and nutrients, drugs and flavors, thus contributing to the regulation of respiration rate and reducing enzymatic browning in fruits [115,122–124]. A chitosan/

methyl cellulose film, and a chitosan/methyl cellulose film incorporating vanillin (vanillin film) as a natural antimicrobial agent (a phenolic aldehyde) was also developed for fresh-cut cantaloupe (*Cucumis melo* L.) and pineapple (*Ananas comosus* L.), improving the microbial control and fruit quality during storage at 10°C [11].

Currently, the chitosan and its functionalization confers to this copolymer new physiological and biological properties with great potential in a wide range of applications in industries such as cosmetology (lotions, hair additives, facial and body creams), food (coating, preservative, antioxidant, antimicrobial), biotechnology (chelator, emulsifier, flocculant), pharmacology and medicine (fibers, fabrics, drugs, membranes, artificial organs), and agriculture (soil modifier, films, fungicide, elicitor) [17,18,47,63,64,67,125–128].

Regarding the agriculture sector, the improved chitosan solubility makes it easy and useful in applications in aqueous solutions at various concentrations [23,24]. The functionalized chitosans developed for controlled drug release can also be used as vehicles for controlled deliveries of fertilizers, pesticides, herbicides, nematicides, and insecticides in soil [22]. Coating of seeds and leaves with chitosan derivatives films can also prevent microbial infections and induce genes in plants that may help them resist diseases [22].

In the context of food and packaging industries bio-derived polymers have become the main ingredient for the future generation of materials, because the annual consumption of plastic materials has now reached nearly 100 million tons worldwide and continues to grow. It is estimated that 56% of disposable nonbiodegradable plastics used as packaging or containers are generating an alarming environmental problem. Since the 1980s, manufacturers of packaging materials are under pressure to meet varied demands that provide functional improvements with environmentally sustainable and compatible characteristics at low cost. Hence, the efforts are mainly aimed toward the development of new active, intelligent, and bio-based packaging materials for foodstuffs. Bio-based packaging materials deserve special mention because they must be made from renewable sources and because of the urgent need for functional bio-based packaging materials. Active packaging materials are those that change the conditions of the packed food either to extend shelf life, to guarantee food safety, or to improve sensory quality. On the other hand, the intelligent packaging systems should control the conditions of packaged foods to provide information about the food during transport and storage [129].

In this context, compared with other bio-based food-packaging materials, chitosan has the advantage of being able to incorporate functional

substances such as minerals or vitamins and possesses antibacterial activity. Therefore, not only chitosan films have been used as a packaging material for the quality preservation of a variety of foodstuffs but also its derivatives. The water vapor permeability of chitosan films may be moderated by chemical modification with a cross-linking agent.

Chitosan and its derivatives with DD 95% and M_w 21000 Da and lower DD and higher M_w , such as carboxymethylated chitosan, chitosan sulfates, chitosan–arginine, chitosan Schiff base, hydroxypropyl methylcellulose chitosan, and chitosan poly(vinyl alcohol), have shown antimicrobial activity against various microorganisms such as *S. aureus*, *L. monocytogenes*, *P. aeruginosa*, *E. coli*, *Shigella dysenteriae*, *Vibrio cholerae*, *B. subtilis*, and *C. albicans* [2,103,130]; also they have antifungal activity against *B. cinerea*, *Monilinia fructicola*, *R. stolonifer*, *A. niger*, *F. oxysporum*, *C. albicans*, *A. alternata*, and *C. gloeosporioides* [58,60,131–133].

In terms of plastics whose implementation in agriculture (films for greenhouse covers, mulching, low-tunnel cultivation, etc.) has been especially useful, it is important to highlight that the manufacturing of bioplastics represents a real challenge because the biopolymer industry has to meet the uptake of renewable materials in each product and to supply materials for sustainable agriculture now that sustainability is essential for agricultural production [134]. In this context, research is increasingly focused on blends of biodegradable bio-based polymers with synthetic nonrenewable polymers for some agricultural applications. Currently, several biodegradable polymers and biocomposite materials are available under different trade names [135]. Examples include the blends of starch-derived polymers and polylactide (PLA) with polybutylene adipate terephthalate (PBAT). PBAT is a biodegradable plastic manufactured by BASF with the trade name Ecoflex®. In this particular case, the role of PBAT in starch blends is to provide hydrophobicity in the material for better water resistance and mechanical properties. To date, a few biopolymers, such as cellulose, cereal flour, and sucrose, are among the raw materials used for the blends mentioned, and chitosan has not been reported to be used for this purpose so far.

CHARACTERIZATION OF CHITOSAN DERIVATIVES MOLECULES

A relevant fact worth pointing out is that the size, functional groups, and structure of a novel compound is difficult to determine. Therefore, in addition to classical analysis, it is important to make an integral characterization

using advanced technologies such as ^1H -NMR, HPLC, UV/vis, Fourier transform infrared spectroscopy (FTIR), MALDI TOF MS, scanning electron microscopy (SEM), viscometry (determination of molecular weight), and rheology, because these current techniques all provide precise and reliable information about the degrees of polymerization (DP), degrees of deacetylation (DA), proportion and position of the functional groups of the biopolymer functionalized, particle sizes, and viscoelastic properties [136].

Therefore, the FTIR analysis allows confirmation of the functionalization. In other words, the IR spectra display the characteristic absorption band of the different groups. For example, the bands at 3354 and 3286 cm^{-1} correspond to $-\text{NH}$ stretching; the band at 2870.97 cm^{-1} results from the stretching of CH_3 ; meanwhile the bands at 1651 cm^{-1} and 1585 cm^{-1} are attributable to the stretching and bending vibrations for the amide I ($-\text{NH}_2$) in the acetylated units and CH_3 , respectively [20,88,91,93,137].

In a recent report, a chitosan-g-octyl gallate was synthesized in order to improve hydrophobicity and antimicrobial properties plus antioxidant properties. This chitosan derivative exhibited an increased absorption in the 2850–2930 cm^{-1} region, which is ascribed to the symmetric and asymmetric stretching of methylene groups. The presence of carbonyl signal was observed at 1716 cm^{-1} , evidencing the grafting of octyl gallate as illustrated in Figure 2.7 [20].

The IR spectra of chitosan catechin exhibited a strong absorption in the region 3200–3500 cm^{-1} due to the increase of aromatic and nonaromatic stretching hydroxyl groups of catechin; signals observed at 1650 cm^{-1} , 1590 cm^{-1} , and 1380 cm^{-1} are attributed to the NH-bending of the glucosamine unit [93]. The FTIR spectrum of chitosan hexyloxyphenol showed changes due to the NH stretching (3300–3500 cm^{-1}) and NH bending (1590–1650 cm^{-1}) apart from the alkyl stretching region of 2800–2950 cm^{-1} due to hexyloxyphenol [88]. In another study, the chitosan incorporated with green tea (*Camellia sinensis*) showed peaks between 3500 cm^{-1} and 3000 cm^{-1} , corresponding to stretching vibration of free hydroxyl and the asymmetric and symmetric stretching of the NH bonds in amino group, respectively. Another change was observed at 1700 cm^{-1} , ascribable to carbon-to-oxygen ($\text{C}=\text{O}$) stretching within the carboxylic group, and one peak at 1660 cm^{-1} correlated to carbon-to-carbon ($\text{C}=\text{C}$) stretching within the aromatic ring, which indicates that the functional group of phenolic compounds were detected [63].

Unlike FTIR, the ^1H -NMR spectroscopic technique allows determining the degree of deacetylation or degree of grafting made onto chitosan

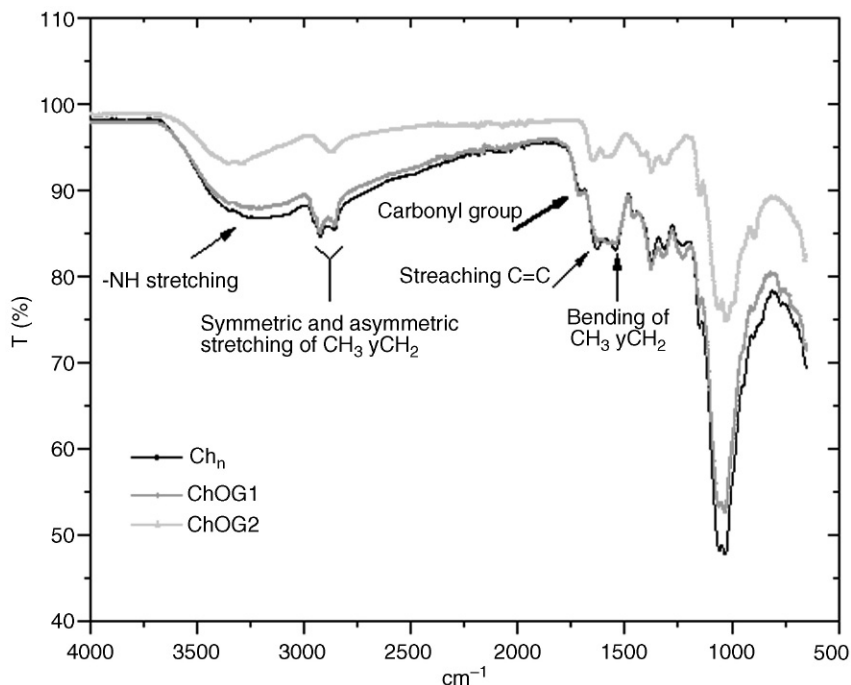


Figure 2.7 ATR-FTIR Spectra of Chitosan (Ch), Chitosan-g-octyl Gallate 4.22% Molar (ChOG1), and Chitosan-g-octyl Gallate 11.75% Molar (ChOG2).

molecules. The spectra of derivatives differ essentially by the presence of characteristic signals of the protons of the glucosamine residues, methyl protons of the *N*-acetyl groups, and protons of molecules or groups (i.e., the methyl and methylene protons of the hexyl chain or alkyl chain). These intensity proton signals (integral value) are used to calculate the grafting and deacetylation percentage; the ^1H -NMR spectra of native chitosan shows a peak at 2 ppm for the CH_3 protons from the acetylated glucosamine residues, meanwhile, the peaks for the C-2 to C-6 protons appear between 3 ppm and 4 ppm [88,97].

Thus, in the case of chitosan-g-octyl gallate, the ^1H -NMR spectra displayed signals at 1–1.2 ppm, which correspond to the aliphatic hydrogens of the alkyl side chain; however, it is important to point out that the signal of aromatic hydrogens are not able to be found because of the overlapping caused by the formation of monomers and polymers of octyl gallate grafted onto chitosan as it is shown in Figure 2.8 [19,82]. In a longer chain gallate such as dodecyl gallate, the ^1H RMN spectra confirmed the mentioned behavior [97].

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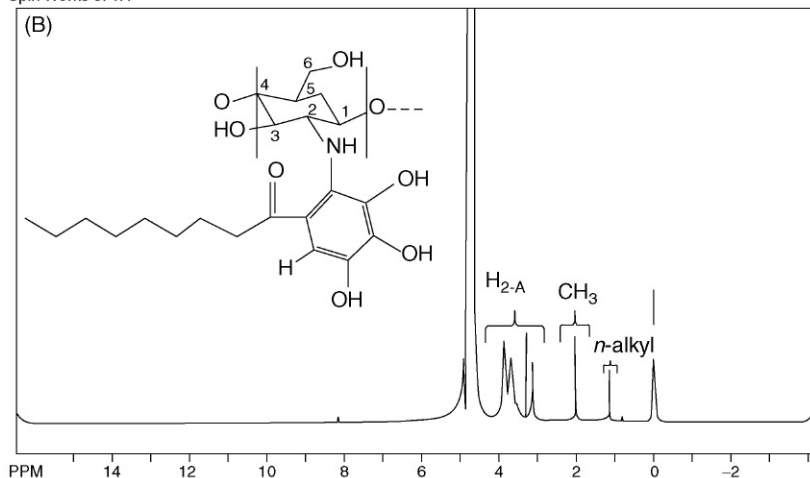
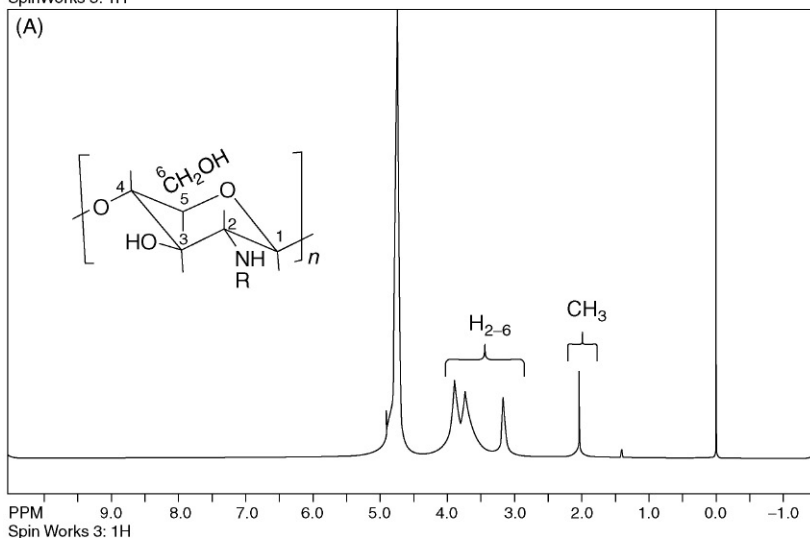


Figure 2.8 ¹H RMN Spectra of (A) Chitosan and (B) Chitosan-g-octyl Gallate.

Thermal analysis is a simple technique that may predict whether the modified molecule obtained is stable under specific temperatures. For example, chitosan/oligo L-lactide has a higher decomposition temperature than native chitosan [138]; on the other hand, chitosan-poly lactide has lower degradation temperatures [139].

Viscosity is an important physical property in determination of chitosan molecular weight (M_w), especially for commercial applications. Higher M_w chitosan produce highly viscous solutions, which may not be desirable

because lower viscosity chitosans may facilitate its handling for industrial purposes.

The MALDI TOF MS (matrix-assisted laser desorption/ionization time-of-flight) is equipment used to identify a molecule and determine its chemical profile structure by analyzing the mass and the charge of its ions. This technique allows knowing the residue distribution in chitooligomers of same DP as a function of DA.

Rheological properties characterization must be involved in any development of new materials in that it provides useful information not only about flow behavior but also the linear viscoelastic properties whose impact is reflected in the prospect of material performance. Hence, the shear-thinning character and viscoelastic properties of a functionalized polymer can be related in terms of structure, the inter- and intramolecular interactions, conformation of the polymeric chains, as well as their molecular weight and colloidal properties, which are important features for future studies in bio-based materials with potential in the formulation of edible films or coatings and applications in food preservation. So, the solution behavior of functionalized chitosan is essential to envisage its potential applications. In particular, the linear viscoelastic properties of aqueous solutions of chitosan are closely related to their colloidal structure, while shear rate dependence of viscosity is associated with its structural modifications [16,19,123].

CONCLUDING REMARKS

Research devoted to chitin and chitosan since as early as the 1930s has generated relevant information about their properties and, as could be expected for such a wide range of chitosan modifications, research reports vary in nature from basic scientific studies to practical and commercial topics. However, it is noteworthy that the latest reports on chemically or enzymatically functionalized chitosan open up a new generation of high-value materials for a wide range of specific applications. The main interest for this trend has been the pressure for innovations in environmentally friendly materials for packaging. This means the development of bio-based materials from sustainable resources that have good mechanical and barrier properties to protect the product.

Chitosan blends and functionalized chitosan (i.e., chitosan derivatives) are extremely promising materials for bio-based active films or coatings preparations for improved or new properties for preserving and extending the shelf life of foods. More research is needed, however, for food-packaging

applications. Furthermore, much of the current research and development of product applications for new chitosan biomaterials have been carried out in laboratory or pilot plant environments. Chitin and chitosan are still being modified; but as several researchers have underlined in recent reviews, a deeper scientific knowledge is needed of these novel compounds in order to understand the mechanisms of their biological activities. The chemical reactions mechanisms, which occur during chitosan modifications, are of great interest in order to understand not only its biological function but also its potential applications. Hence, the development of each particular novel chitosan derivative should be accompanied by details of molecular modification because it is increasingly relevant to generate well-defined functionalized chitosan polymers in order to correlate their biological, physical, chemical, and rheological and stability characteristics with their bioactive properties.

The potential of nanotechnology for engineering materials with novel and unique properties in the agricultural, food, and packaging sectors is high. Still, concern over the use of nanoparticles in food as well as its manipulation using nanotechnologies also remains high. In this context, further research is needed to provide safety data before nanoparticles can be added in food.

In summary, whether chitosan alone, blended, functionalized, or as nanocomposite, this fascinating biopolymer will continue to be explored as a highly promising resource in the production of novel future materials.

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PART 2

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CHAPTER 3

Application of Chitosan in Fresh and Minimally Processed Fruits and Vegetables

Susana Patricia Miranda-Castro

INTRODUCTION

A growing trend around the world is the exploration of new alternatives to control postharvest pathogenic diseases, prioritizing methods that reduce disease incidence and avoid negative and adverse effects on human health from the excessive use of synthetic fungicides. Currently, preservation of fresh and minimally processed foods includes the use of biodegradable, biocompatible, and nontoxic materials to improve food safety and extend the shelf life of these products. In many cases, such as in fruits, their quality will be significantly improved.

Edible coatings formed with safe materials offer several advantages over the synthetics, such as biodegradable and environmentally friendly materials. In addition, some of these coatings have the potential to improve the appearance of foods and retard or inhibit the growth of pathogenic and spoiled microorganisms. However, development of edible coatings must fulfill several requirements to be accepted as an appropriate alternative to synthetic materials. Basically, edible coatings must be water-resistant and stable during cold storage, not causing depletion of O₂ or excessive CO₂ accumulation. They must be minimally permeable to water vapor, improve fruit gloss and appearance, not be sticky, have a good drying performance, maintain fresh product quality without releasing undesired odors, have low viscosity, and be translucent and affordable.

Chitosan (CH) is a polysaccharide obtained from the exoskeleton of crustaceans, such as shrimps and crabs [1]. It has become a potent alternative treatment for extending storage life and controlling decay of fruit and vegetables due to its natural antimicrobial effects and elicitation activities in plant tissue [2–7]. Since the early 1990s, many authors have reported the ability of chitosan to form edible films that have been applied in a variety

of fruits. In addition, researchers have made great efforts to improve their functions by mixing chitosan with different essential oils and plant extracts. However, fruits should be considered individually and hence edible films should be designed like a tailored suit.

In this chapter, some of the advances achieved in the preservation of fruits and vegetables by the application of chitosan and its different modes of application are described.

APPLE

Rose apples (*Syzygium agueum* Alston) cv. Tabtim Chan is an economically important fruit from Thailand and because its peel is thin, it deteriorates quickly with low water retention. Plainsirichai et al. [8] studied the effect of chitosan treatment on the quality of this cv. stored at an ambient temperature of 30°C and 60–65% RH. Results demonstrated that at day 5 of storage, the fruit coated with 2% chitosan had a weight loss of 12.82% and a disease incidence of 14%, which were significantly less than those of the control (22.12 and 24%, respectively). Fruit firmness of the rose apples treated with 2% chitosan was significantly higher than that of the control (5.92 kg/cm₂ and 4.12 kg/cm₂, respectively). Chitosan was also shown to protect rose apples against disease infection.

ASPARAGUS

Green asparagus (*Asparagus officinalis* L.) is eaten worldwide as a popular fresh vegetable due to its high nutritional value. However, its respiration rate makes it particularly susceptible to postharvest perish. Asparagus is also easily infected by the *Fusarium* species causing the stem and crown to blight, resulting in decay during postharvest. The antifungal activity and effect of chitosan solutions of high molecular weight (H-chitosan) with a 423 mPa s and deacetylated degree of 95.6%, low-molecular weight (L-chitosan) with a viscosity of 20 mPa s and deacetylated degree of 96.1%, and carboxymethyl chitosan (C-chitosan) with a viscosity of 92.5 mPa s and deacetylated degree of 91.0% adjusted to pH 5.6 were tested *in vitro* and *in vivo* as coatings on postharvest green asparagus were evaluated by Qiu et al. [9].

As tested *in vitro*, L-chitosan and H-chitosan inhibited the radial growth of *Coniothyrium concentricum*, with a remarkable effect at a concentration of 4 mg/ml, and totally inhibited spore germination at a concentration of 0.05 mg/mL, indicating that chitosan derivatives were either fungistatic

or fungicidal. The antifungal indexes of L-chitosan, H-chitosan, and C-chitosan were 89, 74, and 22%, respectively. Coated asparagus did not show any apparent sign of phytotoxicity and maintained good quality over 28 days of cold storage (2°C and 95% RH), according to the weight loss values and general quality aspects. Results indicate that chitosan could act as an attractive preservative agent for postharvest green asparagus owing to its antifungal activity and its ability to stimulate some defense responses during storage.

AVOCADO

Avocado fruit (*Persea americana* Mill) plays an important role in human nutrition due to its nutritional properties such as oleic, palmitic, linoleic and palmitoleic acids, a trace amount of stearic acid, vitamins A, B, C, E, K, and high fiber content. Avocado fruit has high economic value; however, major postharvest losses are encountered throughout the supply chain due to an anthracnose disease caused by the fungus *Colletotrichum gloeosporioides*. This fungus affects the fruit quality, marketability, and shelf life of avocados during marketing [10,11]. Postharvest losses due to anthracnose can reach up to 80% unless fruits are treated with prochloraz (N-propyl-N-[2-(2, 4, 6-trichlorofenoxi) etil] imidazol-1-carboxamida) at postharvest [12]. At the packinghouse, after cooling the fruit, they are commonly treated with this nonsystemic fungicide as a first defense mechanism [13]. Acidified 200 ppm prochloraz + 50 mM citric acid are effective for the control of postharvest diseases on both “Fuerte” and “Hass” [14].

However, there is a need for safer methods to control postharvest decay development due to an increase in consumer concern regarding food safety and demand for organically produced fruit. In addition to this, development of fungicide resistance [15] and growing global pressure on the fruit industry to lower the associated environmental pollution footprint have resulted in the need to search for natural novel products to replace prochloraz fungicide application at the postharvest stage. Several techniques have been used for storage of climacteric fruits to retard the rate of ripening after harvest and thus extend the shelf life. These include controlled/modified atmosphere (CA/MA) storage, modified atmosphere packaging (MAP), and/or application of special skin coatings. In all these cases, the resulting atmosphere modification (i.e., lowering the O₂ and/or increasing the CO₂ concentrations in the storage environment) has been shown to be helpful in extending the storage life of several perishable produce [16].

Edible coatings can create a modified atmosphere, similar to that of MAP, their effectiveness being a function of coating permeability and fruit respiration. Temperature control is an important step because it can affect the permeability as well as the rate of fruit respiration. Higher temperatures increase fruit respiration rates. Coating can be formulated from different materials including lipids, resins, polysaccharides, proteins, and synthetic polymers.

Salvador et al. [17] used chitosan coating on avocado fruits and improved the storage life to 24 days at 3–10°C and to 6 days at 27–29°C.

Qiuping et al. [18] treated avocado fruits firstly with 1-MCP for 24 h, followed by treatment with an aqueous solution of 2% chitosan, and then storage at 20°C and 85–90% RH for ripening assessment. Ethylene and carbon dioxide production, firmness, weight loss, color index, polygalacturonase (PG) activity, and polyphenol oxidase (PPO) activities were assayed during storage. The results indicated that avocado fruits treated with 100 nL/L of 1-MCP for 24 h and/or treated with 200 mg/L chitosan reduced ethylene and carbon dioxide production and weight loss, delayed firmness loss, maintained the peel color, and suppressed the PPO and PG activity. Chitosan coating with 1-MCP treatment had a better effect because it delayed the ripening time for 12 days.

Bill et al. [19] investigated the *in vitro* antifungal effect of gum arabic (GA) (10%), aloe vera (AL) (2%) and chitosan (CH) (1%) alone or in combination with thyme essential oil (1%). Chitosan and thyme oil, and AL and thyme oil [1:1 or 3:1 v/v] showed positive fungicidal effects while AL, CH, GA and GA and thyme oil [3:1 v/v] showed fungistatic effects on mycelial growth of *C. gloeosporioides*. They recommend the combination of chitosan and thyme oil [3:1 v/v] as a suitable alternative to the currently adopted prochloraz applications in controlling anthracnose disease in these fruit during storage.

BANANA

Banana (*Musa acuminata*) is a popular fruit worldwide due to its flavor, texture, nutritional value, and convenience of being easy to peel and eat [20]. It contains a lot of nutrients and minerals that are beneficial to health. Its vitamin C content, which is regarded as a familiar antioxidant, is 15%. Bananas are usually harvested before being fully mature for domestic consumption. Usually they are stored at room temperature. However, banana is susceptible to several diseases resulting in massive and extensive postharvest losses during transportation and storage [21]. Anthracnose is a latent infection where fungal spores infect immature banana in the field but symptoms do not

appear until the peel blemishes, as black or brown sunken spots of various sizes on fruit that may bear masses of salmon-colored acervuli with their associated conidia on the fruit peel after ripening [22,23]. Other fungal diseases, including crown rot caused by a fungal complex, *Colletotrichum musae*, *Fusarium* spp., and *Lasiodiplodia theobromae*, are also a major cause of postharvest diseases. Infection was higher when combinations of virulent pathogens attacked the fruit. Postharvest storage conditions may accelerate the development of the disease.

Wattanakorn [24] showed that chitosan (1%) coating of banana fruit prolonged storage life for up to 27 days at 13°C with 90–95% RH. Win et al. [25] studied cinnamon extracts (CE) and chitosan. Their hypothesis was that application of two or more substances combined could enhance the fungitoxic potential. CE alone showed fungistatic and fungicidal activity against anthracnose and crown rot pathogens when spraying them on banana cv. Embul prior to storage while chitosan coating (1%) delayed disease development and ripening. In this study, the combination of treatments was not effective for controlling crown rot and prolonging fruit quality. CE and chitosan were not compatible and the combination reduced the effectiveness of chitosan for delaying ripening.

In another study, Maqbool et al. [26] studied a composite coating for controlling postharvest anthracnose in banana. The composite coating was produced with two polysaccharides, Arabic gum (AG) and chitosan (CH). AG has received the highest toxicology safety status conferred by the Joint FAO/WHO Expert Committee on Food Additives [27]. However, its use as an antifungal agent has not been reported yet. In this study, results showed that chitosan alone had the greatest effects against *C. musae* in *in vitro* experiments; however, less effectiveness was observed when applied on banana. *In vitro* as well as *in vivo* results of antifungal effect of AG used alone confirmed that AG had no fungicidal effects against *C. musae*, but AG showed better results than chitosan coatings in terms of preserving bananas. The edible composite coatings of 10% AG incorporated with 1% chitosan was found to be the best in controlling the incidence of anthracnose both *in vitro* and *in vivo*; the composite showed synergistic effects and the greatest potential to control anthracnose in bananas and maintained quality for up to 33 days.

Suseno et al. [28] also worked with chitosan edible emulsion coating in order to improve shelf life in Cavendish bananas. The effect of different degrees of deacetylation (DD) of chitosan (70, 80%) at various chitosan concentrations (1, 1.5, 2% w/w) on weight and vitamin C loss were investigated. In this study, the effect of the presence of the emulsifier

triethanolamine (TEA) was also examined. Weight loss and vitamin C decreased with increasing chitosan concentration and degree of deacetylation. Addition of the emulsifier did not significantly influence these two parameters. In summary, 2% (w/w) chitosan with DD of 80% was proved to be the most suitable coating.

BROCCOLI

In recent years, the demand for broccoli (*Brassica oleracea* L.) for fresh or ready to eat salad consumption has increased greatly [29]. However, the main problem that makes fresh-cut broccoli a highly perishable product is the ease of microbial growth [30]. Cutting or slicing operations greatly increase tissue damage and cause the release of intracellular contents [31]. The release of cellular substrates supports and increases the activity of pathogenic and saprophytic microorganisms.

Native microflora evolution (mesophilic and psychrotrophic) of fresh-cut broccoli was followed during refrigerated storage. Also, the effects of chitosan coatings combined with bioactive compounds (BC), such as bee pollen, ethanolic extract of propolis, pomegranate dried extract (*Punica granatum*), and resveratrol (3, 40, 5-trihydroxy-trans-stilbene), or essential oils (EO), including tea tree (*Melaleuca alternifolia*), rosemary (*Rosmarinus officinalis*), clove (*Syzygium aromaticum*), lemon (*Citrus limonum*), origanum (*Origanum vulgare*), calendula (*Calendula officinalis*), and aloe vera (*Aloe ferox*), on the survival and growth of *Escherichia coli* and *Listeria monocytogenes* inoculated in broccoli were evaluated [32]. Chitosan and CH plus EO/BC significantly inhibited the growth of mesophilic and psychrotrophic bacteria, and also controlled *E. coli* and *L. monocytogenes* survival. The application of chitosan coatings alone and enriched with EO/BC did not introduce deleterious effects on the sensory attributes of minimally processed broccoli.

CARROT

Storage rot is a significant problem for New Zealand exporters of fresh carrots (*Daucus carota* L.). Cheah et al. [33] identified *Sclerotinia sclerotiorum* as the most common of seven fungal storage pathogens during monthly monitoring of cool-stored carrots. Cheah et al. [34] investigated the *in vitro* effects of chitosan at 1, 2, and 4% (w/v) on this fungus.

The effect of chitosan coating on sclerotinia rot of carrots (*D. carota* L.) held at 22°C was also investigated. Carrot roots were coated with chitosan

solutions (2 or 4%) and inoculated with mycelial plugs of *S. sclerotiorum* culture. After 5 days of storage, chitosan at both rates reduced significantly the incidence of rot (from 88 to c. 28%) and also the lesion size (from 26 to c. 12 mm) of the rot on roots.

Molloy et al. [35] also measured the susceptibility of carrots to this storage pathogen after roots were treated postharvest with a 0.2% (w/v) chitosan hydrolysate (number average degree of polymerization = 7). The hydrolysate did not affect the radial growth of *S. sclerotiorum* colonies on potato dextrose agar plates, but it reduced the frequency and size of rots compared to the untreated controls when applied to carrots 3 days before inoculation. However, when carrots were treated at time zero with either chitosan hydrolysate or high molecular weight chitosan and then inoculated at intervals over the next 5 days, there was a decline in *Sclerotinia* infection, with the hydrolysate showing a greater effect than the high molecular weight chitosan. These results suggest that the chitosan treatments induced host resistance to the pathogens.

In fresh carrots, edible coatings are mainly applied to control dehydration as a moisture barrier or surface moisturizer, reducing the transport of soluble particles and gas [36,37]. Different formulations of edible coatings have been tested to reduce surface dehydration to reduce white discoloration of carrots and microbial proliferation that are the main postharvest problems of this commodity [38,39]. Among them, hydrophilic biopolymers such as cellulose derivatives [40,41], casein [39], and hydrophilic polypeptides have been successfully applied in experimental studies of ready-to-eat carrots.

Among the wide variety of minimally processed vegetables available, the consumption of fresh-cut carrots as ready-to-eat snacks or as salad vegetables is increasing in popularity [42]. However, sales are hindered because of rapid deterioration during storage, and shelf life is usually limited to 7–8 days even with refrigerated storage at 5°C [43]. High respiration rate, development of off-flavors, acidification, loss of firmness, discoloration, and microbial spoilage are some of the major problems associated with minimally processed carrots.

Durango et al. [44] showed that ready-to-eat carrots covered with an edible coating made of 4% starch yam, 1.5% chitosan, and 2% glycerol and placed on polystyrene trays kept their freshness appearance and microbial growth was reduced during storage at 10°C for 15 days.

Simões et al. [45] found similar results by using a combined application of an edible coating containing chitosan under moderate O₂ and CO₂ levels. The coating maintained the sensory quality of carrot sticks while enhancing the phenolic content.

Chitosan has been studied as a successful edible coating material for whole fruits and vegetables but application in fresh-cut produce has been explored only in the past decade. Evaluation of chitosan to extend the shelf life quality of shredded carrots is largely unexplored, though there are studies on the beneficial effects of chitosan coating on carrot slices [46] and sticks [45] using the dip technique. Several disadvantages are associated with the dip technique. One is the need for a large amount of coating solution to complete immersion of the commodity leading to longer preparation time. Reuse of the coating is not possible due to quality alteration of the solution.

Pushkala et al. [47] studied the efficacy of the chitosan powder coating technique as an innovative alternative to spray and dip coating methods for quality maintenance of shredded carrots. This new technique also includes low-density polyethylene (LDPE) as a suitable packaging material. They studied the effect on the physicochemical parameters, bioactive compounds, and microbial and sensory quality of carrot shreds stored in macro perforated LDPE packages. Samples treated with 0.2% chitosan powder had significantly lower weight loss, higher moisture retention, and better vitamin C retention throughout the 10-day storage period.

CITRUS

Spain is the main producer of citrus, including oranges, mandarins, and lemons, in Europe. Citrus and other kinds of fruit can be stored for long periods once they have been coated with chitosan [48]. However, postharvest fungal disease caused by *Penicillium digitatum* severely affects the storage and transportation of oranges, particularly during “ethylene degreening” [49]. Notably, increasing the fruit’s natural defense capabilities through induction of resistance by pre- or postharvest application has made a significant contribution to nonfungicidal *Penicillium* decay control in citrus fruit [50–52].

Two varieties of citrus, Fortune mandarins (*Citrus reticulata*) and Valencia oranges (*Citrus x sinensis*), coated with chitosan were studied by Galed et al. [53]. They evaluated its effect on maturity, decay, and damage, and its fungistatic effect through the magnetic resonance imaging (MRI) technique, to monitor the process of ripening and decay, finding that the dissolution of the chitosan on the mandarins and oranges produced excellent results in terms of percentage of weight loss, MRI, and visual appearance. It preserved Fortune mandarins for 6 weeks and Valencia oranges for 22 weeks. The fungistatic effect of chitosan in the two varieties was notable because both species had a healthy appearance after 8 weeks in a damp storage room.

Navel orange (*C. sinensis* L) fruit is a subtropical fruit of high commercial value on the international fruit market and is commonly eaten fresh. *Penicillium italicum* and *P. digitatum* are considered to be the most important postharvest pathogens and are the causal agents of blue mold and green mold, respectively [54,55].

Mandarin orange (*Murcott tangor*) is a citrus fruit hybrid (*C. reticulata* and *C. x sinensis*). Also called the temple orange, its thick rind is easy to peel and its bright orange pulp is sour-sweet and full-flavored. Chien et al. [56] covered this fruit with low molecular weight chitosan (LMWC, MW = 15 kDa) and high molecular weight chitosan while studying decay and maintenance of its quality. While 0.1% LMWC coating substantially slowed decay of fruit stored at 15°C by over 20%, 0.2% LMWC was more effective in controlling the growth of fungus on citrus fruits caused by *P. digitatum* and *P. italicum*. LMWC coating improved firmness, titratable acidity, ascorbic acidity, and water content in fruit stored at 15°C for 56 days.

Zeng et al. [57] studied the disease resistance and reactive oxygen species (ROS) induced metabolism chitosan fresh navel oranges treated with a 2% chitosan solution for 1 min and inoculated with *P. digitatum* and *P. italicum* stored at 20°C and 85–95% RH. They reported that treatment with 2% chitosan significantly reduced disease incidence and lesion diameter compared to the control. This treatment significantly improved activities of peroxidase (POD) and superoxide dismutase (SOD), and the levels of glutathione (GSH), and hydrogen peroxide (H_2O_2) inhibited the activity of catalase (CAT).

Arnon et al. [58] tested the efficacy of a newly developed polysaccharide-based edible bilayer coating comprising carboxymethyl cellulose (CMC) and chitosan in preserving postharvest quality of various citrus fruit, including cvs. Or and Mor mandarins (*C. reticulata*), “Navel” oranges (*C. sinensis*) and “Star Ruby” grapefruit (*Citrus paradise*) after simulated storage and marketing.

The 1.5% CMC and 1.0% chitosan solutions were applied as separate layers, because the CMC/chitosan composite formulation proved nonhomogeneous, whereas application of chitosan upon the CMC layer resulted in a clear, homogenous, and stable coating. Furthermore, the proposed CMC/chitosan bilayer coating has the advantage of combining the beneficial properties of each individual ingredient into a superior coating formulation [59].

Their findings showed that this new bilayer coating met most of the indicated requirements for edible coatings. Various characteristics of this

CMC/chitosan bilayer coating were highlighted, such as how it was water resistant and how it maintained its stability during exposure to cold storage and shelf-life conditions. It did not promote excessive CO₂ build-up or cause anaerobic conditions in the internal atmosphere of the fruit. It improved gloss and appearance as well as not being sticky and having good drying performance; it also maintained the flavor quality of oranges and grapefruits, covered the fruit surface adequately and, being based on polysaccharide materials, was quite inexpensive and economical in use. Finally, it formed a homogenous, translucent coating.

Satsuma orange (*Citrus unshiu*) is a small, seedless, and easy-peeling citrus cultivar well known worldwide because of its sweetness, hardness, and simple cultivation. It is about the size of other mandarin oranges (*C. reticulata*), but smaller than an orange. One of the distinguishing features of the Satsuma is its distinctive thin, leathery skin dotted with large and prominent oil glands, which is lightly attached around the fruit, enabling it to be peeled easily in comparison to other citrus fruits. The Satsuma also has particularly delicate flesh, which cannot withstand the effects of careless handling. The Chinese and Japanese names refer to Wenzhou, a city in the Zhejiang Province of China known for its citrus production. However, it has also been grown in Japan since ancient times, and the majority of cultivars grown in China today were cultivated in Japan and reverse introduced into China in modern times.

Lu et al. [60,61] demonstrated that preharvest application of chitosan oligomers (COS) alone was effective in controlling green mold decay caused by *P. digitatum* in Satsuma orange. When applied at a high concentration (1%, w/v), COS can effectively reduce disease incidence and severity after spraying; it can also decrease spore germination. The low level of COS (0.2%, w/v) showed a minimal toxic effect on spore germination. However, the low concentration of COS showed an equivalent effect with those at high dose in controlling green mold at 4 days of storage when the incubation time was prolonged enough. However, the combination of pre-COS (1%, w/v) and *Rhodosporidium paludigenum* as biocontrol showed a more effective decay control than any other treatment.

Cháfer et al. [62] investigated chitosan-based coatings incorporating bergamot oil, tea tree oil, and thyme oil on orange cultivar Navel Powell as preventive and curative treatments, against *P. italicum*. The greatest antifungal effectiveness against this fungus was obtained for preventive treatments with coatings containing tea tree oil. Curative treatments were less effective and, in this case, the coatings with thyme oil showed the greatest antifungal

activity. The quality parameters of oranges obtained throughout postharvest suggest that the coatings did not have a notable effect on fruit development, but they slightly enhanced the water vapor barrier properties, especially when they contain essential oils.

Control of postharvest diseases by chitosan or essential oil treatments seems to occur through two different mechanisms: a direct germicide effect on pathogens and an indirect effect by inducing defense mechanisms in fruit tissue [63–65]. Satsuma mandarin (*C. unshiu* Marc.) cv. Miyagawa wase was studied by Shao et al. [66]. They investigated the antifungal ability of chitosan 95% deacetylated combined with clove oil on mycelial growth, membrane permeability, and ultrastructure of *P. digitatum*, and the effects of chitosan coating with clove oil on green mold development and defense-related enzymes in artificially inoculated citrus fruit.

They found that chitosan combined with clove oil inhibited mycelial growth more than individual treatments, which was related to the greater release of cellular material and the largest alterations in hyphal morphology of *P. digitatum*. However, compared to chitosan alone, 1% chitosan coatings combined with various amounts of clove oil (0.5, 1, or 2 mL/L) showed no greater ability in controlling decay development on artificially inoculated citrus fruit and that 1% chitosan combined with 0.5 mL/L clove oil appeared to slightly reduce lesion diameter and enhanced the activities of defense enzymes, including chitinase and phenylalanine ammonia-lyase at the later stages of incubation.

DRAGON FRUIT (PITAYA)

Dragon fruit or red pitaya *Hylocereus polyrhizus* (Weber) Britton & Rose is native to Mexico but is now cultivated worldwide, including in Vietnam, Taiwan, Southern China, Israel, and more recently in Thailand, Australia, the United States, and Malaysia. Dragon fruit has gained global attention due to its prominent purple red color and high antioxidant content. The major constraints in dragon fruit production are attributed to biotic and abiotic factors, for example anthracnose disease caused by the fungus *C. gloeosporioides* that can be devastating at the farm level and during storage. In addition, dragon fruit has a short storage life due to high respiration, weight loss, and accelerated ripening, with shriveling of fruit being evident from 8 days following harvest [67].

In a published work Ali et al. [68] evaluated conventional chitosan low molecular weight from crab shell (50 kDa; 75–85% deacetylated)

and submicron chitosan dispersions (SCD) for the control of postharvest anthracnose and quality maintenance of dragon fruit during storage at $10 \pm 2^\circ\text{C}$ and $80 \pm 5\%$ RH for 28 days. The maximum reduction (93.45%) in disease incidence (DI) and disease severity (DS) was recorded with 1% chitosan at 600 nm droplet size. The results suggest that chitosan solutions were effective in controlling the disease, deferred the onset of disease symptoms and slowed down disease progress but had no effects in maintaining the fruit quality.

GRAPES

Commodities like table grapes (*Vitis vinifera* L.) with thin pericarp and succulent flesh are a highly perishable, nonclimacteric fruit. Its shelf life is usually shortened by firmness loss, berry drop, discoloration of the stem, desiccation, and fungal rots resulting in a high decay rate during storage. The main decay is gray mold caused by *Botrytis cinerea* and at times by *Penicillium expansum*. The commercial method to control decay of the table grape fruit is the use of SO_2 during cold storage, either by fumigation or generators [69,70]. Despite its excellent effect in controlling decay and preventing stem browning, SO_2 application is becoming restrictive in many countries.

Table grapes are often cold stored at 0°C soon after harvest, so as to retain fruit quality and delay senescence, and thus reduce decay development. Treatment with chitosan at 0.5 and 1% decreased the spread of gray mold infection from a berry to the closest neighbors both at room temperature and after cold storage [71]. Blue mold was reduced by preharvest chitosan treatment or postharvest UV-C application, and their combination resulted in a synergistic reduction [72].

The combined treatment of reduced doses of chitosan (0.1 and 0.5%) and ethanol (10 and 20%) provided an additive, and at times synergistic decay reduction of artificially inoculated gray mold for table grape berries kept 7 days at 15°C and bunches cold stored for 2 months, and then exposed to 3 days of shelf life [6]. The effectiveness of chitosan to control gray mold infections in artificially inoculated berries and bunches changed greatly according to the acid used to dissolve the biopolymer, and acetic acid performed best [73]. Several physiological changes have also been observed in host tissues treated with chitosan. Table grape bunches with a preharvest spraying with chitosan showed a threefold increase in phenylalanine ammonia-lyase (PAL) activity in the berry skin 24 h and 48 h after the application [71,74]. The treatment with 1% chitosan decreased hydrogen

peroxide production in table grape berries, showing a protective and anti-oxidant effect.

The effectiveness of grapefruit seeds extract GSE 0.1% (commercial product derived from the seeds and pulp of grapefruit) and chitosan 1% to control postharvest decay and quality of grape berries cv. Red Globe stored at 0–1°C was investigated by Xu et al. [75]. In this study chitosan and GSE treatments, alone or combined, significantly reduced postharvest fungal rot of the fruit challenged with *B. cinerea*. Grapes treated with GSE and chitosan significantly reduced fruit firmness loss after 4 weeks of cold storage, and the change in grape skin color was slower. The beneficial effect of GSE or GSE with chitosan coating was also clear in delaying rachis dehydration and browning, which is associated with decayed berries. Sensory analyses of the berries resulted in high scores for all analyzed parameters. These results are in agreement with the lower weight loss found in GSE with chitosan-treated berries possibly due to increased sugar concentration as a result of water loss.

The effects of 1 g/L preharvest chitosan spray (PCS) and/or postharvest chitosan coating (PCC) treatments on the quality and physiological response of table grapes cv. Jingxiu was evaluated by Meng et al. [76]. Deacetylation occurred with 90–95% chitosan and at a concentration of 1 g/L, it was used for both treatments. The treated fruit was packaged in plastic boxes, then overwrapped with plastic bags to maintain the relative humidity (RH) at 90–95%, and finally stored at 20 or 0°C. The experiment revealed that preharvest chitosan spray induced the activities of defense-related enzymes polyphenol oxidase (PPO) and phenylalanine ammonia lyase (PAL), thus promoting protection from latent infection of grape fruit pathogens. PCS/PCC treatment showed the best control effect on decay and significantly decreased the weight loss of fruit stored at 20°C.

Meng et al. [77] studied the effects of preharvest spray of *Cryptococcus laurentii* combined with chitosan coating after harvest on decay and quality of table grapes cv. Jingxiu during storage periods. Chitosan 90% deacetylated degree and viscosity 15 cp at a 10 g/L solution was used for coating and the suspension of *C. laurentii* containing concentration 1×10^8 cells/mL was used for spraying preharvest fruits. The fruit was packaged in plastic boxes, then overwrapped with plastic bags to maintain the relative humidity (RH) at 90–95%, and finally stored at 0°C.

In this study, spray application of *C. laurentii* on the surface of table grapes at 10 days before harvest significantly reduced natural decay during the storage periods probably by reduction of initial infection and suppression of saprophytes and pathogens during storage. The results in this experiment

revealed that preharvest spray of antagonist combined with postharvest chitosan coating could more significantly enhance control effect on table grapes decay in storage than pretreatment alone.

The incorporation of minor constituents, such as essential oils, into the polymer matrices such as chitosan can notably change and/or improve the antimicrobial and some physicochemical properties. Bergamot oil is a citrus oil extracted from *Citrus bergamia* whose major chemical compounds are volatiles, such as limonene (32–45%) and linalool (about 10.23%) [78,79].

Sánchez-González et al. [80] analyzed the effect of two different polysaccharide matrices, high molecular weight hydroxypropyl methylcellulose (HPMC) (1%, w/v) and also chitosan (1%, w/v) with or without bergamot essential oil (2% w/v) concentration on the development of physicochemical properties, respiration rate and microbial counts of organic table grapes, cv. Muscatel, throughout 22 storage days at 1–2°C. They found that edible coatings obtained from HPMC and chitosan with and without bergamot essential oil are a good alternative for grape preservation, improving weight losses and fruit firmness, as well as slightly reducing respiration rates. Chitosan coatings containing bergamot oil produced the most effective antimicrobial activity, and showed the greatest inhibition of the respiration rates in terms of both O₂ consumption and CO₂ generation.

Romanazzi et al. [81] wrote a review that summarizes the results published in the field during 2006–2010. They group their approaches as follows: (i) biocontrol agents; (ii) natural antimicrobials; (iii) GRAS type decontaminating agents; and (iv) physical means.

Because the Maillard reaction is complex between an amino compound, often an amino acid, peptide, or protein, and a carbonyl compound usually a reducing sugar, such as glucose, fructose, or lactose, it occurs spontaneously during food processing and storage. The Maillard reaction compounds contribute to the formation of a specific flavor and improvement of some functional food properties [82]. Moreover, some Maillard reaction products have been found to exhibit strong antimicrobial and antioxidant activity [83]. Chitosan possessing an amino group makes it a candidate to take part in the Maillard reaction. Kanatt et al. [84] found that chitosan–glucose complex (CGC), a modified form of chitosan, showed superior antioxidant activity as compared to chitosan/glucose alone.

Gao et al. [85] studied the effects of chitosan–glucose complex (CGC), both at 1%, on the postharvest quality of grapes cv. Muscat Hamburg. All fruits were packed into boxes and sealed in polyethylene (PE) films to maintain humidity and all boxes were stored at 0°C for 60 days and then at 20°C for 3 days. Chitosan–glucose coated treatments significantly decreased

decay, alleviated postharvest deterioration, reduced respiratory intensity, and enhanced antioxidant enzyme activities. The activities of POD and superoxide dismutase (SOD) were higher. CGC significantly alleviated the declines of total soluble solids, ascorbic acid, and titratable acidity. In addition, CGC also effectively maintained hardness, springiness, cohesiveness, and chewiness of fruits and had a beneficial effect on sensory quality of grapes. These results indicated that CGC was a useful strategy for table grapes preservation.

Vasconcelos de Oliveira et al. [86] studied the effects of chitosan obtained from *Cunninghamella elegans* and *Mucor circinelloides* against *B. cinerea* and *P. expansum*, and *Aspergillus niger* and *Rhizopus stolonifer*, respectively, in table grapes [87]. Chitosan obtained from *C. elegans* chitosan strongly inhibited spore germination and induced significant ultrastructural changes in the spores of both *B. cinerea* and *P. expansum*. On the other hand, chitosan from *M. circinelloides* strongly inhibited mycelial growth and spore germination for both fungi tested (*R. stolonifer* and *A. niger*) at all assayed concentrations compared to the control. Chitosan as a coating, especially at 7.5 mg/mL and 15 mg/m¹, combined with low storage temperature delayed the fungal growth and maintained the physical, physicochemical, and sensory characteristics of the grapes over time.

GREEN BEANS

Severino et al. [88] evaluated the antibacterial effects of 1% N-palmitoyl chitosan-based coating incorporating 0.05% (w/v) mandarin EO nanoemulsion alone or in combination with three nonthermal treatments (ozonated water, UV-C and γ -irradiation) against *Listeria innocua* inoculated in fresh green beans (*Phaseolus vulgaris* L). Samples were packaged in 3-mil nylon EVA sterile copolymer bags during storage for 15 days at 4°C. The combination of γ -irradiation in a dose of 0.25 kGy and bioactive coating showed a constant synergistic antimicrobial effect, with a microbial reduction of more than 3 log CFU/g on day 1 of storage, but also a strong residual antimicrobial activity during storage, which brought *L. innocua* population below the detectability limits (50 CFU/g) after 5 days of storage.

GUAVA

Guava (*Psidium guajava* L.) is an important subtropical fruit grown widely in tropical and subtropical regions of the world including China. It is a highly palatable fruit with a rich source of vitamin C [89]. However, harvested

guava can exhibit a high respiration rate and fast ripening that leads to perishability during storage periods.

In order to reduce decay incidence and improve the quality of guava fruit after harvest, Hong et al. [90] studied the effect of chitosan (50–190 kDa, degree of deacetylation) coating on physicochemical characteristics of guava fruit. cv. Pearl. The fruit were treated with 0.5, 1.0, and 2.0% chitosan coatings, and stored at 11°C and 90–95% RH. Treatment with 2% chitosan solution combined with a low temperature inhibited fruit ripening and maintained the quality of the fruit. Chitosan treatment also increased antioxidant activities, significantly reduced firmness and weight loss, delayed changes in chlorophyll, malondialdehyde (MDA), and soluble solids contents, and retarded the loss of titratable acidity and vitamin C during 12 days of storage. This treatment could induce a significant increase in the activities of POD, SOD, and CAT, and inhibited superoxide free radical ($O_2^{\cdot-}$) production.

Bezerra de Aquino et al. [91] used 2% chitosan (DD 85.9%) in combination with 2% cassava starch and essential oils (1, 2, or 3%) from some genotypes of *Lippia gracilis* Schauer (such as Mexican oregano or Spanish thyme) to see the effect on the shelf life of guavas during storage at room temperature for 10 days. These concentrations were the most effective in inhibiting the growth of Gram-positive and Gram-negative bacteria *in vitro*. The incorporation of 1 or 3% mixture of *L. gracilis* essential oils (EOM) into edible chitosan coatings delayed the ripening process, reduced browning, and inhibited color development in guavas.

JUJUBE

Jujube *Ziziphus jujuba* Mill is a native plant of China. Its fruits have been used in traditional Chinese medicine for more than two thousand years. The bioactivities of the polysaccharides in this fruit, such as immunobiological and antioxidant activities, have been reported. The fresh Chinese winter jujube ripens easily and is susceptible to pathogenic infections after harvest, which strongly limits the postharvest life and supply period of the fruit. Infection is mainly caused by *Alternaria alternata* and *Monilinia fructicola*.

Yan et al. [92] evaluated the effects of preharvest foliar sprays with oligochitosan (OCH) (MW, 2 kDa, 5 kDa, 10 kDa, and 85% DD) at concentrations of 0.3, 0.7, and 1.0 g/L on postharvest diseases, ripening, and quality of the Chinese jujube fruit during storage. Mechanisms involved in the fruit defense system as influenced by the OCH spray were also investigated.

OCH sprays significantly reduced postharvest natural decay and delayed skin color changes and firmness decline in the jujubes during storage at 0°C and 80–90% RH. The main defense-related enzymes, including POD and β -1,3-glucanase, were enhanced, and SOD activity, ascorbate peroxidase activity, and glutathione content increased, but CAT decreased.

Chitosan (DD 90% and molecular weight 350 kDa) at 1.5 and 10 mg/mL concentrations was studied by Wang et al. [93] to evaluate its effect against *P. expansum* and natural decay on jujube fruit stored for 70 days at 0°C. Disease incidence and lesion size was the result of inhibition of spore development, germination, germ tube elongation, and mycelial growth by chitosan in a concentration dependent manner. They also found that the plasma membrane of *P. expansum* collapsed significantly after chitosan treatment. Chitosan coating hardly had any significant effect on the changes of weight loss, soluble solid contents, titratable acidity, and vitamin C as storage time increased. Chitosan also affected *P. expansum* on jujube fruit.

LITCHI

Litchi (*Litchi chinensis* Sonn.) is a high commercial value tropical fruit in the international fruit market. However, within 2 or 3 days after harvest its pericarp becomes desiccated and turns brown; it decays and its flavor is lost. Zhang et al. [94] treated the fruit with 1 or 2% chitosan coating 1 h after dipping in 0.1% thiabendazole (TBZ), and then stored at 4°C and 90% RH. They measured changes in browning due to anthocyanins, flavonoids, total phenolic contents, polyphenol oxidase (PPO), and peroxidase (POD) activities. The application of chitosan coating delayed changes of contents of anthocyanin, flavonoid, total phenolics, and the increase in PPO activity, and partially inhibited the increase in POD activity that is associated with tissue browning.

Jiang et al. [95] examined the effects on Litchi fruit treated with 2% chitosan solution and then stored for 20 days at 2°C and 90–95% RH, prior to shelf life evaluation at 25°C and 80–90% RH. Changes in polyphenol oxidase (PPO) activity, anthocyanin concentration, color index, eating quality, and concentrations of total soluble solids and titratable acidity were measured. The effects of chitosan coating on disease incidence were also evaluated. Chitosan coating delayed the decrease in anthocyanin content, the increase in PPO activity, and the changes in color index and eating quality; chitosan also reduced the decrease in concentrations of total soluble solids and titratable acidity, and partially inhibited decay.

Caro et al. [96] found that chitosan solution at pH 1.3 with citric acid gave better results in terms of less weight losses of litchi, and it was also demonstrated that browning was related to dehydration rate during storage, and the fruit pericarp pH.

Ducamp-Collin et al. [97] tested the effect of dipping two litchi cultivars with different pericarp composition into a solution based on citric acid (600 g/L) and chitosan (0.75 g/100 mL). The aim was to determine the mode of action on the cultivars in terms of maintaining the pericarp's red color during storage at 4°C and 90% RH. The chitosan-citric acid treatment increased the shelf life of the fruit by at least 3 weeks, compared to the untreated control that browned rapidly. The activities of PPO, POD, and anthocyanins changed depending on the cultivar and were greater after dipping.

Lin et al. [98] studied litchi treated with 1% chitosan solution and stored at ambient temperature to see the effect on the respiratory behavior and quality of the fruit under static and dynamic storage process states. Chitosan coating restrained the respiration of litchi and produced less bioheat. As a result of respiration and transpiration, the pericarp's temperature of litchi with coating was lower than that of the uncoated fruit and the surrounding environment at all times. It was found that the side of the chitosan coating on litchi, exposed to air, was more uniform and closely packed like a barrier; the other side, which was in contact with the pericarp, had higher roughness and better transport properties. As a result, the chitosan reduced the respiration and transpiration of fruits during storage.

MANGO

Mango (*Mangifera indica* L.) is considered the most popular fruit among millions of people in Asia, particularly in India where it is deemed to be the choicest of all indigenous fruits. Because of its delicious taste and high caloric value, it is ranked as one of the best fruits in the international market. Because of its diverse nature and vast growing area, it suffers from a number of diseases. Some of them take a heavy toll on the crop and are limiting factors in mango production. Among them, anthracnose caused by *C. gloeosporioides* is the most prevalent and serious malady in mango-growing areas.

A talc-based formulation of plant growth promoting rhizobacteria (PGPR) and yeast antagonistic strains with or without chitin amendment was evaluated by Vivekananthan et al. [99] against anthracnose in this

fruit. A preharvest application of *Pseudomonas fluorescens* (FP7) with chitin formulation at monthly spray intervals through aerial spray significantly reduced the pre- and postharvest anthracnose incidence.

Restaurants and consumers like sliced mango for convenience of serving and consumption. However, minimally processed foods are typically stored between 4°C and 8°C. Whole mango fruits exhibit chilling injuries if stored at temperatures under 13°C for several days.

Sliced mango was treated by Chien et al. [100] with aqueous solutions of 0, 0.5, 1, or 2% chitosan (95–98% deacetylated and viscosity 630 mPa s); mango slices were placed into plastic trays, overwrapped with PVDC film, and then stored at 6°C. They evaluated sensory qualities of taste, color and water loss. Chitosan coating delayed the loss in sensory quality, and extended the shelf life.

Nanomultilayer coating, consisting of five nanolayers of 0.2% pectin solution and 0.2% chitosan solution, was applied on mango cv. Tommy Atkins. The coating was characterized in terms of permeability to water vapor, oxygen, and carbon dioxide. After 45 days of storage in plastic boxes at 4°C and 93% RH, mass loss, soluble solids, and titratable acidity were evaluated [101]. The values reported for WVP, O_2 P, and CO_2 P were $0.019 \pm 0.005 \times 10^{-11}$ g/(Pa sm^2), $0.069 \pm 0.066 \times 10^{-14} \pm$ g/(Pa sm^2), and $44.8 \pm 32 \times 10^{-14}$ g/(Pa sm^2), respectively. Coated mangoes presented a better external appearance, a less dehydrated surface, apparently without fungal growth, and a much more preserved flesh.

The Nam Doc Mai Mango, also commonly known as the Thai Mango, was studied by Panupong et al. [102]. They evaluated the effects of different molecular weight (65,000, 240,000, and 410,000 Da) coating solutions on the postharvest qualities of this cv. The solutions were freshly prepared and others were prepared and stored for 14 days. Once the mangoes were covered, they were stored at 25°C for 15 days. The most appropriate MW of chitosan for prolonging fruit shelf life was 240,000 Da, and the nonrefrigerated coating solution was better because its application minimized susceptibility to postharvest disease. Mango fruits coated with this chitosan had 15 days of shelf life and low anthracnose incidence.

The antimicrobial property of elemental silver (Ag⁺) has been studied extensively and it has had more reported applications in medicine than any other inorganic metal ion, while at the same time having shown no harm to human cells. Silver can be used for management of plant pathogens in a relatively safer way as compared to synthetic fungicides [103,104] as it displays multiple modes of inhibitory action.

Silver nanoparticles (AgNPs) were synthesized at 95°C [104] by using chitosan as the reducing agent and stabilizer with the aim to evaluate the chitosan–AgNP composite for its antifungal activity against *C. gloeosporioides* *in vitro* and its efficacy in reduction of anthracnose disease in mango cv. Alphonso. *In vitro* conidial germination indicated that chitosan–AgNP composite exhibited significantly higher antifungal activity against *C. gloeosporioides* while *in vivo* assay showed that anthracnose was significantly inhibited by chitosan–AgNP composite.

The lactoperoxidase system (LPOS) has been described as an excellent system for fighting pathogenic microorganisms because of its broad antimicrobial spectrum. This enzyme system has shown a bactericidal effect on Gram-negative bacteria and a bacteriostatic effect on Gram-positive bacteria. In addition, it has antifungal and antiviral activity. This system generates intermediate antimicrobial products such as hypothiocyanite (OSCN-) and hypothiocyanate acid (HOSCN). These highly reactive products inhibit microorganisms by oxidation of the sulphhydryl groups of microbial enzymes [105–108]. In this respect, chitosan (>90% DDA viscosity 500–2000 cps) could be used to stabilize LPOS antimicrobial activity for a long time, and it may also delay fruit ripening. Mohamed et al. [109] studied the effectiveness of LPOS linked to chitosan (0.5, 1, and 1.5 g) in order to extend the postharvest preservation of mangoes cv. Kent).

In this study, chitosan showed antimicrobial activity, which had been strengthened by the LPOS. Chitosan, at 1% concentration containing LPOS, was sufficiently effective against microbial contamination and enabled a delay in fruit ripening without altering quality.

MUSHROOMS

Shiitake mushroom (*Lentinus edodes*) is highly perishable and tends to lose quality immediately after harvest. Its shelf life is short because of its high respiration rate; it has a tendency to turn brown because it has no physical protection to avoid water loss and microbial attack [110].

Chitosan–glucose complex (CGC), a modified form of chitosan, has shown excellent antioxidant activity. Jiang et al. [111] evaluated the effect of CGC on the microbiological and postharvest quality of this vegetable covered with 1% chitosan solution (deacetylated 95%, and viscosity 630 mPa s) for 16 days at $4 \pm 1^\circ\text{C}$ and 95% RH. Their results indicate that treatment with CGC coating maintained tissue firmness, inhibited respiration rate increase and reduced microorganism counts. In addition,

CGC coating also delayed changes in the ascorbic acid and soluble solids concentrations. Sensory evaluation proved the efficacy of the CGC coating by maintaining the overall quality of shiitake mushroom during the storage period.

PAPAYA

Papaya (*Carica papaya* L.) is one of the most important fruit crops grown in tropical and subtropical regions of the world. Being a climacteric fruit, papaya has a short postharvest life. Postharvest diseases greatly reduce the papaya's storage life, and the fungus *C. gloeosporioides* is one of the most serious problems because of favorable environmental conditions for this pathogen and the acquired resistance to synthetic fungicides.

Anthracnose disease restricts papaya exports to overseas markets. Field conidial inocula of *C. gloeosporioides* originate from dying infected petioles of the lower leaves [112]. Conidia released by rain splash are carried by air currents to developing fruit [113]. If moist conditions persist for a few hours, the conidia develop appressoria from which infection pegs penetrate the fruit skin. Infections remain latent until the fruit ripens [114] and symptoms become evident only during the postharvest period.

Bautista-Baños et al. [115] evaluated *in vitro* and *in vivo* the effect of different chitosan concentrations (0.5, 1.5, 2.5, and 3.0%) and aqueous extracts of custard apple leaves and papaya leaves and seeds (AEPS) on *C. gloeosporioides* development. Mycelial growth of *C. gloeosporioides* *in vitro* was completely inhibited by chitosan concentrations of 2.5 and 3.0% during the 7-day incubation. Papaya fruit treated with 0.5 or 1.5% chitosan and the combination of 1.5% chitosan with AEPS had the least infection (60%), while the lowest disease severity index obtained was from fruit treated with 1.5% chitosan. Firmness was the physiological parameter most affected by the treatments.

Sivakumar et al. [116] evaluated some combined applications of 1% chitosan solution and 2% sodium bicarbonate and 3% ammonium carbonate in the control of anthracnose, and the quality, physical attributes, and marketability of papaya fruit after 14 days at 13.5°C and 95% RH and 2 days under simulated market conditions (25°C, 75% RH). The most significant treatment in reducing anthracnose was the combination of chitosan and ammonium carbonate with a disease severity index of 0, indicating complete absence of disease. Overall quality was significantly better in fruit subjected to a combined treatment with chitosan and ammonium carbonate. Also,

using the same treatment, the highest firmness values and the lowest weight loss were recorded.

Eriany-Raqueeb et al. [117] evaluated the *in vitro* (1.5, 2.5, and 3.5%) fungicidal effect of calcium incorporated or combined with 0.75% chitosan on *C. gloeosporioides*, and storage life and quality of papaya. Chitosan had the greatest effect against *C. gloeosporioides* in both *in vitro* and in disease incidence percentage. The results indicated that 2.5% combined with 0.75% chitosan inhibited spore germination and significantly inhibited mycelial growth. The percentage of anthracnose incidence was significantly controlled (5.6%) using 2.5% of calcium combined with chitosan. This treatment extended the shelf life of up to 33 days with papaya keeping quality attributes.

In other studies, chitin extracted from locally available prawn waste was used to prepare *N*, *O*-carboxymethyl chitosan. The most effective chitosan formulation (0.1–3%) to inhibit radial mycelial growth was selected via a series of experiments on potato dextrose agar. The selected effective chitosan concentration was then tested on papaya cv. Rathna. *In vitro* studies revealed complete inhibition of radial mycelial growth of the pathogen at 1% chitosan and above. Chitosan at a concentration of 1% significantly reduced disease incidence and severity on papaya fruit. Chitosan at a concentration of 1% improved fruit firmness after ripening, protected the fruit from decay, and kept the fruit quality at an acceptable level (with 80% marketability) throughout the storage period.

Hewajulige et al. [118] studied the antifungal activity of irradiated chitosan (5, 10, 25, 50, 75, 100, and 150 kGy) in solution and in powder on two fungal isolates from papaya cvs. Red Lady and Rathna and the efficacy to extend the storage life of papaya. Results indicated no fungal growth on any of the isolates. Eighty percent marketable quality fruits were observed in Rathna papaya while 70% marketable quality fruits were observed in Red Lady subjected to irradiated chitosan treatment and stored at 13.5°C and 95% RH for 14 days followed by 2 days at ambient temperature (28°C ± 2°C).

In other studies, Ganga et al. [119] examined the mode of action of chitosan (72% DDA) solution 0.1–3% (w/v) in controlling anthracnose disease in this same cv. They evaluated the antifungal activity, film-forming ability, and defense mechanism. Chitosan at 1% and above achieved complete inhibition in spore germination of *C. gloeosporioides* after 74 h. The observations on CO₂ concentration revealed that chitosan-treated papaya was significantly higher than the control 2 h after the treatment. Results of

chitinase assay indicated that papaya peel had significantly higher chitinase and β -1,3-glucanase activities when subjected to chitosan.

The *in vitro* and *in vivo* fungicidal activity of chitosan was also studied by Ali et al. [120] directly on *C. gloeosporioides* and on papaya fruits. Chitosan at 1.5 and 2.0% concentrations showed a fungistatic effect with 90–100% inhibition of the fungal mycelial growth. *In vivo* studies showed that chitosan coatings on papaya not only controlled the fruit decay but also delayed the onset of disease symptoms by 3–4 weeks during 5 weeks of storage at $12 \pm 1^\circ\text{C}$ and slowed down the subsequent disease development. However, when leaving the fruits to ripen at ambient temperature ($28 \pm 2^\circ\text{C}$), chitosan at 2.0% was less effective than 1.5% in controlling disease and also delayed the ripening process by maintaining the firmness levels, soluble solids concentration, and titratable acidity values during and after storage.

Ali et al. [121] elucidated the potential of 95% deacetylated chitosan coatings on the extension of the storage shelf life of cv. Ekotika II and also evaluated the influence of different concentrations (0.5, 1.0, 1.5, and 2.0%) of chitosan coatings on the gaseous exchange and quality attributes of the papaya fruit during cold storage. Chitosan provided an effective control in reducing weight loss, maintained firmness, and delayed changes in the peel color and soluble solids concentration during 5 weeks of storage. The titratable acidity declined throughout the storage period, though at a slower rate in the chitosan coated fruit as compared to the control.

Through stimulating defense-related enzymes [122], the control of anthracnose in papaya by applying chitosan at different concentrations (0.5, 1.0, 1.5, and 2.0%) during controlled storage for 15 days was studied. The fruit treated with 1.5 and 2.0% chitosan showed no visible symptoms at all until the end of the experiment and the activities of defense-related enzymes POD, chitinase and β -1,3-glucanase, and total phenolic compounds were all significantly enhanced.

Zahid et al. [123] investigated the antifungal activity of conventional chitosan and chitosan-loaded nanoemulsions against anthracnose with isolates from different tropical fruits. Results of the present study suggest that chitosan-loaded nanoemulsions were more effective in controlling disease, deferred the onset of disease symptoms, and trimmed down the disease progress. Chitosan-loaded nanoemulsions showed better results compared to conventional chitosan treatments. Chitosan at 2% concentration with a droplet size of 200 nm showed the best results in terms of *in vitro* parameters on *C. musae*, while chitosan at the same concentration with 600 nm droplet size showed the best results in two isolates of *C. gloeosporioides*.

Chitosan-loaded nanoemulsion treatments did not show any detrimental effects on the quality of fruits, but conventional chitosan achieved better results in maintaining the quality of fruits for up to 28 days of storage as compared to chitosan-loaded nanoemulsions. Chitosan in the form of nanoemulsions showed better antifungal effects as compared to its conventional form.

Studies on papaya fruits coated with chitosan at four concentrations (1.0, 1.5, 2.0, or 2.5%) and dipped in calcium chloride solution at four levels (1.0, 1.5, 2.0, or 2.5%) were carried out by Chutichudet et al. [124]. In these experiments fruit were stored under ambient temperature (27°C, 80% RH). Results showed that fruit treated with chitosan, irrespective of the concentration, had less fruit weight loss after 6 days of storage and fruit treated with 2.5% chitosan concentration had maximum fruit firmness and delayed red color appearance on the fruit skin.

PEACH

Extending the shelf life of fresh peach (*Prunus persica*) using edible coating is reported to be beneficial to preserve and maintain the freshness of peach due to its short postharvest life at room temperature and its high susceptibility to pathogens.

Chitosan and oligochitosan efficacy at concentrations from 0.1 g/L to 5.0 g/L on spore germination, mycelial growth, and disease control of *M. fructicola* [125] was evaluated. Chitosan and oligochitosan were effective in inhibiting mycelial growth and spore germination *in vitro*, with the inhibitory effect being concentration dependent. The inhibitory effect of both agents was more obvious on mycelial growth than on spore germination. The effectiveness of both treatments on brown rot control in peach fruit stored at 25°C was similar. When the concentration was low, the effect of oligochitosan was significantly better than chitosan. However, at the higher concentration, the antifungal effect of chitosan was almost the same as oligochitosan.

Various studies have reported that oligochitosan treatments also elicit production of hydrogen peroxide, including PAL and POD, and regulate gene expression of β -1,3-glucanase and chitinase. In this regard, Zengxin et al. [126] investigated the effects of 0.5 and 5 g/L chitosan, and 0.5 and 5 g/L oligochitosan on fruit quality, resistance induction and the transcript expression of defense and antioxidant enzymes. Chitosan and oligochitosan were effective in controlling brown rot and the control effect increased

with the treatment concentrations from 0.5 or 5 g/L. It was also found that chitosan and oligochitosan treatments enhanced firmness and acidity, while decreasing total solid solubles during storage at 4°C for 4 days. It was found that the two antifungal substances significantly increased POD and CAT activities in general. Additionally, it was found that the activities of β -1,3-glucanase and chitinase markedly increased in the treated fruit.

Elbarbary et al. [127] studied the antioxidant activity of carboxymethyl chitosan (CMCS) from different molecular weights obtained by gamma radiation. The possible use of CMCS as antioxidant and preservative coating by dipping treatment in peach fruit at ambient temperature was also part of the investigation. The coating of peach with irradiated CMCS, especially at 20 kGy and 30 kGy, prolonged storage life 3–7 days at ambient temperature, keeping good peach color without spoilage, controlling brown rot, and decreasing toxic products such as malondialdehyde content.

PEAR

Alternaria kikuchiana Tanaka and *Physalospora piricola* Nose are two kinds of fungal pathogens of pear (*Pyrus pyrifolia*) fruit in storage. Using these two fungi as subjects, this study [128] aimed to evaluate the differences between chitosan and oligochitosan as fungicides and as elicitors by comparing the inductive effect of enzyme activities on the host. In this study, chitosan (90% deacetylation and average molecular weight of 360 kDa) diluted to a series of 0.1, 0.3, 0.5, 0.7, 1.0, 2.0, and 5.0 g/L, and oligochitosan (6 kDa) dissolved in sterile distilled water at 25 g/L, were tested on spore germination, germ tube elongation, and mycelial growth. Spore germination and germ tube elongation of both fungi were significantly inhibited by chitosan and oligochitosan with concentration dependent mode, although oligochitosan generally showed better inhibitory effect than chitosan, especially on *P. piricola*.

Treatments reduced disease incidence and inhibited the lesion expansion of the two fungi on pear fruit. The activities of PPO and POD in flesh around a wound in pear fruit were first raised and then decreased after 24 h. Chitinase and β -1, 3-glucanase activities increased with storage time in the flesh around the wound in control pear fruit.

In recent years, biological control with antagonistic microorganisms has been considered the most promising alternative method for the inhibition of postharvest diseases, especially for the control of invading pathogens [129].

According to Rosenberger et al. [130] *P. expansum* is the most important pathogenic fungi of the pear, which is responsible for substantial economic losses. The effect of the combination of chitosan, *C. laurentii*, and calcium chloride (CaCl_2) on postharvest decay of pear was studied by Yu et al. [131]. The study was conducted to investigate the potential of chitosan (DD deacetylation of 90% and the intrinsic viscosity 12 cps) alone or in combination with this antagonist and CaCl_2 in controlling blue mold *in vitro* and *in vivo* in Asian pear Nakai cv. Shuijing. The degree to which chitosan inhibited the survival of *C. laurentii in vitro* was dependent on the concentration, with the higher the concentration, the higher the inhibition. Therefore, 0.5% chitosan in combination with *C. laurentii* inhibited *P. expansum*. CaCl_2 (1.0% w/v) alone showed little antifungal activity; however, when it was added to the suspensions of *C. laurentii*, and with the mixture of *C. laurentii*, fungal inhibition improved.

Numerous studies have examined the effects of calcium in fruit firmness and decay after harvest, but few researchers have focused on changes in the composition of the cell walls of the fruit during storage. In another study [132], chitosan and calcium treatments were applied to provide an adequate barrier to retard the loss of malic acid and enzyme activities and elucidate the possible relationship between malic acid metabolism and gene expression and to compare the efficacy and the importance of the quality preservation treatments in pear cv. Huang Guan. Changes in titratable acidity content of peels and pulp and malic acid (MA) content in pulp were similar. These variables were higher in peels than those in pulp. CaCl_2 and chitosan treatments significantly inhibited acid degradation in pulp and peels. Treatments also delayed ripening and senescence, thereby improving pear quality. The 0.2% chitosan treatment combined with 2% CaCl_2 delayed MA degradation and significantly attenuated enzymatic activity, but the gene expressions were not significantly impacted under both treatments.

PLUM

Plums (*Prunus mume*) are highly perishable fruits because they ripen quickly after harvest. Depending on the variety, plums may have a shelf life of 2–6 weeks, even if stored at 0°C. Storage at low temperatures effectively delay fruit ripening and extend the postharvest life of plums, but the benefits may be limited by the development of disorders associated with cold injuries including internal browning, translucency of meat, and redness.

Liu et al. [133] treated plums with 40 mM ascorbic acid solution, 1.0% chitosan solution, and the combinations of both treatments. Samples were stored at 5°C and 90% RH for 20 days. Quality parameters and enzyme activity were performed in the pulp for determining shelf life and the market value of fruit. The application of 40 mM ascorbic acid in combination with 1% chitosan markedly delayed color changes, extended storage life, and delayed firmness loss in peach. PME and PG activities in combined solutions were lower than the control; also, induced activities of antioxidant enzymes, including SOD, POD, and CAT, were significantly higher, while O₂ production was significantly reduced.

POMEGRANATE

Granada (*P. granatum* L.) is a fruit native to Iran and its surroundings. The edible parts of the shells (called arils) constitute approximately 50% of the weight of the fruit and consist of 76–85% juice and 15–24% seeds. Fresh granada juice contains 85% humidity, and substantial amounts of sugar, pectin, organic acids, polyphenolic compounds, anthocyanins, amino acids, and minerals.

Varasteh et al. [134] evaluated the effect of 1 and 2% chitosan (average molecular weight) and storage temperature on the content of total and individual anthocyanins of pomegranate placed in baskets and stored at 2 and 5°C at 90 ± 5% RH for 45 days. The storage time and temperature as well as the chitosan treatment influenced total anthocyanin content. The obtained result showed that with progressing storage duration, total anthocyanin content declined throughout the storage period in all treatments. At the end of storage time, the total anthocyanin content recorded for the controls stored at 5°C was the lowest (553 mg/L), and at 2% chitosan, coated pomegranate fruit had the highest anthocyanin content (868 mg/L).

POTATO

Fusarium dry rot (*Fusarium sulphureum*) is one of the most important diseases of potato (*Solanum tuberosum*), affecting tubers in storage and seed pieces after planting.

Sun et al. [135] determined the effect of 1% chitosan (DD 92.2%) treatment on potato tuber resistance to dry rot and its possible mechanism. Chitosan treatment at 0.25% significantly reduced disease lesion

diameter. It also enhanced POD and PPO activities in potato slices. A slight increase in PPO activity was observed in the treated slices challenged by *E. sulphureum*. Chitosan treatment caused a more progressive and significant increase in β -1,3-glucanase and PAL activities on tissues challenged by *E. sulphureum*. The content of flavonoids enhanced gradually after 2 days of treatment. However, the content of lignin increased continually during the period of experiment. Different induced effects were found at different times after treatment with the same concentration of chitosan. The result suggested that the defense response induced by chitosan was associated with incubation time, which may be needed for host reception, signal transduction, and related gene repression. The levels of flavonoid compounds and lignin in the tuber tissue with chitosan treatment increase, suggesting that flavonoid compounds, as a type of phytoalexin, can directly kill the fungal pathogen.

RADISH

Pushkala et al. [136] studied a powder coating technique using purified chitosan (CH) (molecular weight 161 kDa and degree of deacetylation 85%) and chitosan lactate (CL) for shelf-life extension of shredded radish (*Raphanus sativus*). Also, a macro perforated low-density polyethylene (LDPE) was selected as the appropriate package. They evaluated physicochemical parameters, bioactive compounds, microbial quality, consumer acceptance, and marketability. Minimally processed radish was coated with two forms of 0.2% chitosan, purified chitosan and chitosan lactate. Physiological loss in weight (PLW) was significantly lower in CH and CL samples (3.33 and 3.19%), respectively, compared to the control (4.83%). With respect to the physicochemical parameters, bioactive compounds, microbial quality, and sensory acceptability throughout the 10-day storage period, chitosan-treated radish shreds were significantly better than the control shreds. Chitosan coatings were also beneficial in maintaining a higher product quality during the storage period.

RAMBUTAN

Rambutan (*Nephelium lappaceum*) is a highly perishable and nonclimacteric tropical fruit, which is appreciated for the exotic flavor of its juicy and sweet pulp. Despite its increasing demand, commercialization is limited by moisture loss during storage and pericarp browning.

Martínez-Castellanos et al. [137] evaluated the effect of *Lactobacillus plantarum* alone or in combination with 2% low molecular weight chitosan (171.4 kDa, 15% degree of acetylation) on quality and color retention of rambutan fruits stored at 25°C and 10°C, and 75% RH for 10 and 15 days, respectively. Fruits were packaged in clamshells with six holes of 0.9 cm inner diameter at each side of the box. The development of the microorganisms was evidenced by viability analyses and lactic acid production. The application of *L. plantarum* significantly improved color retention and reduced weight losses. The lactobacilli, alone or in combination with chitosan, preserved fruit quality characteristics such as firmness, total soluble solids, and titratable acidity. The lactobacilli application on rambutan produced acidification of pericarp and avoided browning, thereby preventing desiccation due to biofilm formation.

RASPBERRY

Raspberries (*Rubus idaeus*) are fruit with high metabolism that makes them highly perishable, impairing their storage and shelf life. Tezotto-Uliana et al. [138] studied the effects of preharvest and postharvest chitosan application. Postharvest application was carried out by dipping the fruit in three different solutions (0.5, 1.0, or 2.0%) for 5 min and storing them at 0°C and 90% RH. The authors reported that preharvest chitosan at 2% and postharvest application of 1 and 2% were able to retain key raspberry quality attributes for 15 and 12 days, respectively. In this study, fresh berry fruits deteriorated rapidly due to water loss, juice leakage (stem scar injury), gray mold, and ripe rot.

Yang et al. [139] quantified the phenolic compounds in blueberry fruit and leaf extracts (BLE), first to evaluate the antimicrobial activity of BLE and then to investigate the effectiveness of a chitosan coating incorporating BLE with a modified atmosphere packaging (MAP) ($3 \text{ kPa O}_2 + 12 \text{ kPa CO}_2$) on fresh blueberries (Lanfeng species). BLE showed antimicrobial effects on all test strains and was especially effective against *E. coli* and *Staphylococcus aureus*. Syringic acid, well known for its antimicrobial activity, was the highest phenolic compound in leaf extracts. Adding blueberry fruit and leaf extracts into chitosan coatings further enhanced the function of the chitosan coating with respect to delaying decay and weight loss, and maintaining total phenolic content and radical scavenging activity during storage, especially when working together with MAP.

RED BELL PEPPER

El Ghaouth et al. [140] studied the effect of chitosan on the infection process of bell pepper fruit by *B. cinerea* with particular emphasis on the ability of *B. cinerea* to degrade host cell wall components, namely pectin and cellulose. Bell peppers (*Capsicum annuum*) scars were treated with a chitosan solution (10 mg/mL) and then inoculated with *B. cinerea*. Cytochemical and enzymatic examinations of chitosan-treated bell peppers showed disease development after 7 days of storage, while lesions were visible in untreated fruit after 3 days [141]. Chitosan treatment also restricted the proliferation of *B. cinerea* in bell pepper tissue and markedly reduced the maceration of the host cell wall components, pectin and cellulose. This seems to result from the ability of chitosan to cause severe cellular damage to *B. cinerea* and to interfere with the capability of *B. cinerea* to secrete polygalacturonases.

Poverenov et al. [142] studied the effect of a composite gelatin–chitosan (GL–CH) coating on the quality of pepper fruit cv. Vergasa. The combination of the beneficial features of the two components resulted in notable fruit structure enhancement, inhibition of decay, and better storability. Utilizing the composite coating resulted in a significant extension of bell pepper shelf life for up to 3 weeks under cold storage conditions.

SQUASH

Cifone [143] studied the effect of coating Anquito squashes (*Cucurbita moschata*) from the lower valley of the Rio Negro in the northeast of Patagonian region, with different concentrations of a chitosan extracted from shrimp biowaste of the fishing industry (MW = 391 kDa and 83% DD). The results of this study verified that the optimal chitosan concentration was 0.2% because it provided the best results, maintaining postharvest quality and improving antifungal properties.

Pugliese et al. [144] studied the effect of the chitosan with different molecular weights (281, 122, and 56 kDa) on the postharvest decay, and the quality maintenance of the coated Anquito squash during 5 months of storage. At the end of the storage periods, no sign of visual fungal decay was observed in squashes treated with 122 and 56 kDa. These treatments were also the best in reducing fruit weight loss. However, from the point of view of other nutritional and quality parameters, chitosan of 122 kDa was the best in maintaining quality and prolonging the storage life.

STRAWBERRIES

In order to slow down metabolism and reduce deterioration prior to transport or storage, strawberries (*Fragaria x ananassa* Duch) are cooled to 0°C after harvest. Their postharvest life can be extended by several techniques combined with refrigeration such as calcium addition that plays a major role in maintaining the quality of various fruit and vegetables, including strawberries. Increasing the calcium content in the cell wall of fruit tissue can help to delay softening and fungal growth and reduce physiological disorders.

Hernández-Muñoz et al. [145] studied the effects of several treatments comprising postharvest calcium gluconate (CaGl) dips, chitosan coating and the incorporation of CaGl into the coating formulation, on fungal decay and fruit quality attributes of strawberries (*Fragaria x ananassa* Duch.), stored at 20°C for up to 4 days and 70% RH. Calcium dips were effective in decreasing surface damage and delaying both fungal decay and loss of firmness compared to untreated fruit. No sign of fungal decay was observed in fruit coated with 1.5% chitosan which also reduced fruit weight loss. Chitosan coatings markedly slow the ripening of strawberries as shown by their retention of firmness and delayed changes in their external color. To a lesser extent titratable acidity and pH were also affected by coatings. Although the addition of CaGl to the chitosan coating formulation did not further extend the shelf life of the fruit, the amount of calcium retained by strawberries was greater than that obtained with calcium dips alone, thus resulting in increased nutritional value of the strawberries.

Vargas et al. [146] evaluated the effect of chitosan–oleic acid edible coatings on the physicochemical and sensory quality, fungal decay, resistance to water vapor transmission, and respiration rate of cold-stored strawberries cv. Camarosa. They applied a high molecular weight chitosan (deacetylation degree of 82.7%) at 1% and oleic acid at 0, 1, 2, and 4%. Strawberries were dipped in the film-forming solutions for 15 s, dried by natural convection for 1 h at 20°C and packed in 750-mL perforated PET boxes stored at 4°C. Edible chitosan-based coatings did not induce significant changes in quality of strawberries in cold storage, but resulted in better preservation of the mechanical properties and color. The coatings improved resistance to water vapor transmission; however, it was generally felt that the coatings decreased the sensory quality of strawberries.

Hernandez-Munoz et al. [147] studied the effect of chitosan coatings combined with calcium gluconate (CaG) on strawberries cv. Camarosa in

terms of quality attributes during refrigerated storage. Strawberries were treated with 1 and 1.5% chitosan acetate solution (CS), with or without the addition of CG. Assessment of the treatments were based on their effects on fungal decay, respiration rate, quality attributes, and the visual appearance of strawberries stored for 6 days at 10°C. No sign of fungal decay was observed during the storage period for fruit coated with 1.5% CS with or without the addition of CaG. This compound helped extend the shelf life of strawberries by inhibiting fungal decay and maintaining fruit firmness when used in combination with a low concentration of chitosan. A chitosan concentration greater than 1% masked any effect of CaG. Sensory analysis of strawberries based on visual appeal also showed that chitosan coatings delayed fruit senescence associated with color changes and dehydration. Incorporation of CaG increased the nutritional value of fruit without altering visual appeal.

Vu et al. [148] carried out *in vitro* tests with natural antifungal compounds (red thyme, oregano extract, limonene, and peppermint) in order to assess their capabilities against molds and total flora, isolated from strawberries. Also, the antimicrobial compounds were evaluated as emulsion-based coatings for their specific antifungal properties on fresh strawberries. A functionalized chitosan palmitoyl chloride was emulsified with limonene and mint essential oils to evaluate the shelf life of the treated strawberries. Limonene, peppermint, and red thyme were efficient for preserving the quality of strawberries for 12 days. Red thyme was considered the most efficient antifungal because fruit treated with this EO had the lowest decay level, but formulations based on modified chitosan containing limonene in the presence of Tween 80 provided better preservation of strawberries than other formulations.

Film-forming dispersions (FFD) were prepared by Perdonés et al. [149] with 1% high molecular weight chitosan (deacetylation degree of 75.6%) and 3% lemon essential oil. Stand-alone coatings were characterized in terms of water vapor permeability (WVP) and antimicrobial activity *in vitro* against *B. cinerea*. The dispersions were applied to cold-stored strawberries cv. Camarosa and stored on PET trays. The physicochemical properties, fungal decay, and respiration rate of strawberries were determined throughout cold storage at 5°C and 95% RH. No fungal growth on film coated CH-LO-M plates was observed. The antifungal activity of chitosan was enhanced by the addition of lemon oil, and significant differences were due to the FFD microfluidification showing a high anti-Botrytis effect.

Strawberries are an important source of bioactive compounds due to high levels of vitamin C, vitamin E, β -carotene, and phenolic compounds such as anthocyanins. The anthocyanins are responsible for the red color of strawberry and are quantitatively the most important phenolic compounds. Strawberries possess a high level of antioxidant activity and therefore have a consequent beneficial effect on the maintenance of consumer health.

Gol et al. [150] assessed the efficacy of 1% carboxymethylcellulose (CMC), 1% hydroxypropyl methylcellulose (HPMC), and 1% chitosan (CH) on the quality of strawberry stored at 11°C and 70–75% RH. The results indicated that CMC, HPMC, and CH coatings had a beneficial impact on the quality retention of strawberry fruit during storage. The coatings of CMC (1%) + CH (1%) and HPMC (1%) + CH (1%) were the most effective approach for quality preservation of strawberry fruit since they resulted in a significant delay in weight loss, decay percentage, total solid solubles, pH, acidity, and ascorbic acid content, and had positive effects on maintaining higher concentrations of total phenolics and total anthocyanins.

In the same year, Wang et al. [151] studied the effect of chitosan treatments on the deterioration of strawberry ex Rozier Earliglow, stored at 5°C and 10°C for 12 days. After 9 days of storage at 10°C, decay percentages for chitosan-coated strawberries (0.5, 1.0, and 1.5 g/100 mL) were 25.3, 15.7, and 6.9%, respectively, while in the control berries the incidence was 95.2%. The effect of chitosan in reducing microbial growth was more evident for longer storage periods and at higher storage temperature. Chitosan-treated samples maintained higher levels of flavonoids than the control samples at the end of both 5°C and 10°C storage.

Lopes et al. [152] investigated the effect of silicon and chitosan as alternative control for postharvest rot of strawberries cvs. Camarosa and Oso Grande caused by *B. cinerea*. Chitosan and potassium silicate applications were performed at the preharvest stage. Chitosan application to plants in the field and in combination with postharvest dipping of fruits was an effective strategy for controlling gray mold in fruits in cv. Oso Grande but was not effective in cv. Camarosa. Potassium silicate was not effective for controlling gray mold in any of the cultivars tested.

SWEET CHERRY

Sweet cherry (*Prunus avium*) is a perishable fruit, and during storage it can undergo severe postharvest decay. Fruit can be damaged by *Monilinia laxa* (brown rot), *B. cinerea* (gray mold), and with a lower incidence, *A. alternata*

(*Alternaria* rot), *P. expansum* (blue mold), *R. stolonifer* (Rhizopus rot), and *Cladosporium* spp. (green rot) [153].

Romanazzi et al. [154] determined that chitosan 1% and hypobaric treatments (0.50 atm) were effective in reducing decay of sweet cherries; the interaction between chitosan and hypobaric treatments was significant in reducing brown rot and gray mold in the first year, and total rots in the first and second year.

Feliziani et al. [155] evaluated the *in vitro* ability of oligosaccharides, benzothiadiazole, chitosan, calcium plus organic acids (COA), and nettle extract as alternatives to fungicides and fenhexamid to inhibit the growth of *M. laxa*, *B. cinerea*, *R. stolonifer*, and *A. alternata*. The *in vitro* ability of chitosan to reduce mycelial growth of the microorganisms was comparable to those obtained with the synthetic fungicide fenhexamid.

They also evaluated the effectiveness of preharvest and postharvest applications of these and other resistance inducers, such as laminarin, potassium bicarbonate, fir extract for the control of brown rot, gray mold, Rhizopus rot, Alternaria rot, blue mold, and green rot during the storage of sweet cherries at room temperature ($20 \pm 1^\circ\text{C}$) and at cold temperature ($0.5 \pm 1^\circ\text{C}$). No statistical differences were noted among these treatments. Compared to the control, the application of nettle extract, chitosan, oligosaccharides, benzothiadiazole, and COA reduced the sweet cherry total rots of 66, 56, 46, 34, and 27%, respectively. The most effective treatments in controlling postharvest total rots of sweet cherry were chitosan and potassium bicarbonate at concentrations ranging from 4 g/L to 26 g/L.

SWEET PEPPER

One of the most important commercial vegetables is sweet pepper (*C. annuum* L.). It is a perishable vegetable with a short lifespan and high susceptibility to fungal diseases. Pepper fruits commonly face problems such as postharvest chilling injury when stored below 7°C and dryness associated with rapid weight loss.

Xing et al. [156] reported that sweet pepper treated with chitosan–cinnamon oil coatings showed lower disease incidence and good sensory acceptability. Defense-related enzymes in plants were also induced by chitosan–cinnamon oil coating. The values of electrolyte leakage and malondialdehyde (MDA) content increased in the coating-treated sample during storage time. The atomic force microscopy (AFM) images showed that surface of sweet pepper without coating treatment was rougher than that with

chitosan coating. Authors pointed out that quality maintenance and shelf-life extension of sweet peppers treated with this coating can be considered for commercial application during storage and marketing.

TOMATO

In particular, the consumption of tomatoes (*Lycopersicon esculentum* Mill) in raw form in salads, hamburgers, and fresh juices has increased. They are a good source of ascorbic acid, carotenoids, and folate.

Liu et al. [157] investigated the effect of 2% chitosan (90% DD and viscosity of 15 cp) on the control of gray and blue rots in tomatoes stored at different temperatures. Results indicated that chitosan provided an effective control of both diseases of tomato fruit stored at 25 and 2°C. Chitosan strongly inhibited spore germination, germ tube elongation, and mycelial growth of both fungi. Studies demonstrated that chitosan damaged the plasma membranes of conidia. Chitosan also induced a significant increase in the activities of PPO and PO, and enhanced the content of phenolic compounds.

Badawy et al. [158] evaluated the effectiveness of chitosan with different molecular weights (0.5×10^4 , 3.7×10^4 , 5.7×10^4 , and 2.9×10^5 g/mol) to control gray mold *in vitro* and *in vivo* in tomato fruit stored at different temperatures. For the mycelial radial growth assay, chitosan solutions were added into PDA medium at concentrations ranging from 250 mg/L to 5,000 mg/L. According to the *in vitro* results, 500, 1,000, 2,000, and 4,000 mg/L concentrations were chosen as the optimal ones to carry out the *in vivo* experiment against *B. cinerea*. Enzymatic and total soluble phenolics content assays were also carried out. In these experiments, chitosan treatments significantly reduced fungal decay. At concentrations of 2,000 mg/L and 4,000 mg/L, chitosan showed complete control of the fungus in wound-inoculated fruit. Results also revealed that high chitosan concentrations were correlated with low disease incidence. Chitosan treatment also decreased the activity of PPO and enhanced total protein and phenolic compounds in wounded tomato fruit.

Previous studies carried out by Wang et al. [159] showed that fructose polymer burdock fructooligosaccharide (BFO) isolated from roots of *Arcitum lappa* was able to increase the quantity of volatile organic compounds of *L. esculentum* cv. Shengfen, and the resistance to *B. cinerea*. It was reported that this compound also induced accumulation of salicylic acid (SA) and increased the activities of some defense-related enzymes. In this

research, the effects of BFO on the control of postharvest diseases in tomato fruit and the underlying mechanisms were also investigated with the application of chitosan oligosaccharide (CO) (degree of polymerization 2–20, average weight $\leq 3,000$ Da, with 90–100% deacetylation). Results indicated that both BFO and CO effectively inhibited natural postharvest diseases and reduced disease incidence of *B. cinerea*. BFO increased the mRNA level of genes encoding pathogenesis-related proteins (PRs), such as PR-1a, PR-2a (extracellular β -1,3-glucanase), PR-2b (intracellular β -1,3-glucanase), PR-3a (extracellular chitinase), and PR-3b (intracellular chitinase) and induced RNA accumulation of the PAL gene in this fruit.

In another study Ramos-García et al. [160] evaluated the effectiveness of the combined treatments of chitosan with two essential oils, beeswax and oleic acid in controlling *R. stolonifer* and *E. coli* DH5 α *in vitro*, and on tomatoes at small scale and at semi-commercial levels. Overall, coating applications were better against *E. coli* DH5 α than *R. stolonifer*. Experiments demonstrated that the best coating was that made of chitosan (1%) + beeswax (0.1%) + lime essential oil (0.1%), with no growth of *R. stolonifer* and *E. coli* DH5 α as a result.

The effects of chitosan (deacetylated $\pm 95\%$) with methyl jasmonate (MeJA) against *A. alternata* *in vitro* and *in vivo* were studied by Chen et al. [161]. The results of that experiment showed that the combination of chitosan and methyl jasmonate resulted in a significant enhanced control of *A. alternata* *in vitro* and on tomato. In the inoculated tomato, 0.1% chitosan and 500 mL/L MeJA enhanced the activities of PAL, PPO, and POD, which are considered to play important roles in plant disease resistance. MeJA at all tested concentrations alone, or in combination with chitosan, was able to suppress the growth and germination of *A. alternata* *in vitro*.

CONCLUDING REMARKS

Recently, a great deal of research has focused on producing and applying bio-based polymers. The emphasis on environmentally friendly technology has stimulated interest in biopolymers, which are more versatile and far more biodegradable than their synthetic counterparts. Several of these biopolymers come from agricultural, marine, and microorganism wastes. These biopolymers include starches, cellulose derivatives, chitosan/chitin, gums, proteins (animal or herbal), and lipids. These materials offer the possibility of obtaining thin films and coatings to cover fresh or processed foods to extend their lives. Such is the case of chitosan.

Chitosan is derived from chitin by deacetylation, and it is described in terms of degree of deacetylation and average molecular weight. Its importance resides in its antimicrobial properties in conjunction with its cationicity and film-forming properties. Through the summary of many articles, it can be seen that these two important chitosan parameters often seem inconsequential. The main parameters influencing the characteristics of chitosan are its degree of deacetylation (DD) and molecular weight (MW), which affect its solubility and rheological, physical, and biological properties. Various grades of chitosan are commercially available, which differ primarily in the degree of deacetylation and molecular weight. Different conditions such as type and concentration of reagents, and time and temperature employed throughout processing, can affect the physical characteristics and performance of the final chitosan product. However, both DD and molecular weight can be further modified.

Another important feature of this polymer to consider is the molecular weight and its distribution. The first difficulty encountered in this respect concerns the solubility of the samples and the dissociation of aggregates often present in polysaccharide solutions. It is not enough to mention that chitosan is of high, medium, or low molecular weight; it is also of utmost importance to evaluate the average molecular weight and degree of deacetylation. Molecular weight tells us whether the molecule is small enough and enters the cell triggering defense mechanisms, or if large enough that its function is antimicrobial and as a barrier.

The exact mechanism by which chitosan exerts its antimicrobial activity is still under study. It has been suggested that the polycationic nature of this biopolymer that forms from acidic solutions below pH 6.5 is a crucial factor. Thus, it has been proposed that the positively charged amino groups of the glucosamine units interact with negatively charged components in microbial cell membranes, altering their barrier properties, and thereby preventing the entry of nutrients or causing the leakage of intracellular contents.

Another reported mechanism involves the penetration of low molecular weight chitosan into the cell, binding to DNA and subsequently inhibiting RNA and protein synthesis. Chitosan has also been shown to activate several defense processes in plant tissues and inhibit the production of toxins and microbial growth.

Despite having a broad spectrum of antimicrobial activity, chitosan exhibits different inhibitory efficiency against different fungi, Gram-positive and Gram-negative bacteria, and viruses. Most studies regarding the application

of chitosan on agricultural commodities focus on diseases caused by fungi at pre- and postharvest stages. Unfortunately, few studies have been carried out on bacterial infections or viruses of horticultural commodities.

It is important to consider in *in vitro* tests that they be carried out under similar nutritional conditions of the affected fruit, that is, that the culture media for growing the infecting microorganism should contain extracts, chitosan, and pH of the fruit, because the behavior of microorganisms is different when they are grown under different substrates.

The idea of preserving the fruits should also contemplate that each of them behaves differently. It is well known that they have, among other features, different respiration and ethylene production patterns, skin and chemical properties, and different microorganisms that infect them. Storage according to the horticultural product should also be considered.

When chitosan is applied by dipping the fruit, the contact time of the fruit with chitosan should also be considered; the greater the contact time, the greater the penetration. Therefore, the activity of chitosan might not just be as a physical barrier but also involve a molecular interaction with the fruit.

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CHAPTER 4

Chitosan Protection From Rice Diseases

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INTRODUCTION

Rice (*Oryza sativa* L.) is the staple food for two-thirds of the world population. In addition, it is the agricultural commodity with the third-highest worldwide production, according to data published by FAO [1].

Damage caused by disease is one of the most important limiting factors in rice production. It can cause crop losses up to 100% of total production. Nowadays, application of chemical fungicides is the most common method to control rice diseases, but they can cause harm to both human health and the environment. Therefore, new control strategies that are healthier for people and more environmentally friendly are constantly being sought. In this sense, many scientists have focused their attention on chitosan, a biocompatible natural polymer, renewable, and abundant. Chitosan and its derivatives have been studied intensively for their applications in plant protection. These products have the dual property of inhibiting growth in a great variety of pathogenic fungi [2,3], bacteria [4], and viruses [5], and stimulating resistance mechanisms in plants, which allow them to protect themselves against diseases even in commercial production systems of various crops such as tomato, cucumber, and wheat [6–9]. Chitosan has also been applied to protect rice crops. Data about chitosan action against rice pathogens and response in chitosan-treated plants are discussed in the following pages.

MAJOR RICE DISEASES AND CONTROL METHODS

Approximately 80 rice diseases have been reported in the world [10], and they are caused by fungi, bacteria, and viruses. Fungal diseases are the most important in rice crops, but in appropriate conditions, any pathogenic agent can cause big losses. The most important rice disease in the world is blast caused by the fungus *Magnaporthe grisea* (anamorph: *Pyricularia grisea*) (Figure 4.1). This fungus attacks both plants and panicles. It can cause losses up to 100% of production in favorable conditions. Other important fungal diseases are brown



Figure 4.1 Typical Symptoms of Various Major Rice Diseases. (A) Upper left: rice leaves with typical lesions caused by blast disease (*P. grisea*); (B) Upper right: rice leaf with sheath blight symptoms caused by *R. solani*; (C) Lower left: kernel spotted symptoms in a rice panicle caused by *B. oryzae*, bacteria, and other rice pathogens; (D) Lower right: rice panicle affected by sheath rot disease caused by *S. oryzae*. (All pictures were kindly provided by Ariel Cruz Triana).

spot caused by several fungi, among which *Cochliobolus miyabeanus* (anamorph: *Bipolaris oryzae*) is particularly important. Brown spot reduces the grain number and weight in panicle. It is present in rice-growing areas around the world. Another important disease is sheath rot, caused by *Sarocladium oryzae*. This fungus can often act together with the mite *Steneotarsonemus spinki* and can cause important losses, dramatically reducing kernel weight. Other major rice diseases are sheath blight caused by *Rhizoctonia solani* (anamorph), leaf scald caused by *Rhynchosporium oryzae*, and Pecky rice (kernel spotting) caused by a group of pathogenic fungi, including *C. miyabeanus*, *Curvularia* sp. and *Fusarium* sp.

Bacterial leaf blight and leaf streak caused by *Xanthomonas oryzae* pv. *oryzae* and *X. oryzae* pv. *oryzicola* are the two most damaging bacterial diseases of rice [4]. Additionally, bacterial brown stripe is another important bacterial disease. Meanwhile, “rice tungro disease” and white leaf are the most important rice viral diseases. An important aspect to point out is that infected seeds transmit most of these diseases and usually more than one disease can attack a rice crop simultaneously.

The main methods to control rice diseases are through the use of chemicals and by plant breeding programs in order to achieve resistant cultivars. Plant breeding is an expensive and time-consuming method. Genetic diversity is a basic and effective strategy to prevent rice disease. However, to release a new cultivar may take at least 5 years using biotechnological techniques, while about 10 years are necessary for traditional techniques. However, a new pathotype may appear in a few years, which may affect this cultivar. Therefore, chemical control is the most frequently used method.

Fungicides are usually effective to control rice diseases. However, their use can be inappropriate, because they are expensive, toxic to humans, and can cause severe damage to the environment. They also often have a unique action mode; therefore, pathogens can perform genetic mutations and become resistant to pesticides in a short period of time. Additionally, most pesticides usually do not have a significant effect in promoting plant growth, the germination rate, or improved crop yield. Lasting effects have been shown following chitosan application. Additionally, chitosan acts by different mechanisms to protect the plant. So far in Cuba its production is not expensive and can be achieved from renewable sources such as crustacean shells, fish waste, or from fungal mycelia wastes produced by industrial fermentation processes.

ANTIMICROBIAL ACTIVITY OF CHITOSAN AGAINST RICE FUNGAL PATHOGEN

Chitosan and its derivatives have exhibited effective *in vitro* inhibition on the growth of major rice pathogenic fungi and bacteria. In this sense, it has been established that protonation of the free amino group on an

acidic medium enhances antifungal activity. Studies report that *P. grisea* mycelium growth is totally inhibited by partially acetylated chitosan and is hydrolyzed at a concentration of 1000 and 500 mg/L [11]. Another research group reported that new chitosan derivatives also have antifungal activity against *P. grisea*. They encountered up to 77% mycelial growth inhibition with hexanoyl chitosan at 5 g/L [3]. In addition, chitosan administered at 1 g/L completely inhibited *R. solani* mycelial growth, while colloidal chitin, a neutral form, only inhibited 20%, evidencing the importance of positively charged amino groups [12]. In this sense, another result reported that two types of acid-soluble chitosan caused a 60–91% inhibition in mycelial growth of *R. solani*. This chitosan effect on this pathogenic microorganism was attributed to direct destruction of the mycelium, confirmed by scanning and transmission electron microscopy [13].

Development of other major rice pathogenic fungi has also been inhibited in *in vitro* conditions. Cuban researchers have reported that *S. oryzae* growth was inhibited by up to 35% with chitosan from lobster shell [14]. Furthermore, two chitosan polymers with 90% degree of deacetylation totally inhibited the mycelial growth of *B. oryzae* at 1000 mg/L. Additionally, chitosan showed positive *in vitro* antifungal activity on pathogenic fungi that cause spotted grain in rice, as both mycelial growth and sporulation were inhibited [15]. Factors determining chitosan antifungal activity include degree of acetylation and molecular size, concentration, and the type of fungi. Nevertheless, a tendency toward greater growth inhibition was observed by increasing both degree of deacetylation and molecular size (avoiding solution that is too viscous) of the tested compounds.

Other authors proposed models to explain the antifungal activity of chitosan. Some of them postulate that chitosan application blocks Ca^{2+} accessibility, essential nutrients, and minerals necessary for the growth of filamentous fungi. Others support the hypothesis that chitosan interaction with the plasma membrane interferes with and modifies membrane permeability [16].

On the other hand, it has also been shown that chitosan has direct antibacterial activity against bacterial leaf blight and leaf streak. This activity may be attributed to both membrane lysis and biofilm destruction [4]. Furthermore, it has been confirmed that chitosan destroys the cell membrane, reduces biofilm, and changes the gene expression of *Acidovorax avenae* subsp. *avenae* RS-1, the rice bacterial brown stripe pathogen [17].

INDUCTION OF RICE DEFENSE MECHANISMS BY CHITOSAN

The defense responses induced by chitosan application in rice includes a battery of early and late reactions such as the increase in H^+ and Ca^{2+} fluxes into the cytosol [18], activation of kinases, hypersensitive responses, and the synthesis of jasmonates, phytoalexins, and PR (pathogenesis related)-proteins [19,20].

The induction of defense mechanisms by chitosan in rice has been attributed to the recognition of chitosan/chitin molecules by specific receptors on rice cells. A Japanese group of researchers [21] that works with rice cell suspension has localized these receptors in the plasma membrane of this crop. These receptors have been identified as high-affinity chitin-binding proteins because they also bind to chitin; therefore, these proteins have been labeled as “chitin elicitor-binding protein” [22]. Several chitosan-binding proteins have been isolated, and it is suggested that they are putative receptors for chitosan [23]. In this sense, the degree of deacetylation is an important factor for the high activity of chitooligosaccharides as well as the molecular size, bigger than that of hexamers [23]. In addition, several gene families were regulated in rice cells in response to chitosan application [24,25].

Chitosan recovered rice seeds by increasing the levels of hydrolytic enzymes such as chitinases and β -1,3-glucanase [26,27], which degrade chitin and 1,3 glucans, respectively. It is known that these compounds are major cell wall components in most phytopathogenic fungi and bacteria. In the same experiment, phenylalanine ammonia-lyase (PAL) and chitosanase also increased four or five times in response to chitosan application. PAL is a key enzyme in the phenylpropanoid pathway and in lignin synthesis and accumulation [28]. The highest PAL elicitation on leaves treated with chitosan was observed at the concentration of 1000 mg/L and with hydrolyzed chitosan at the concentration of 500 mg/L, suggesting that chitosan and chitosan oligosaccharides have different mechanisms in the elicitation of rice defenses. An explanation for this might be that the small size of chitosaccharides allows them to pass easily through plant cuticle and interact effectively with the receptors, while the molecular size of chitosan does not permit the same activity. Therefore, interaction of chitosan as a polycationic molecule with negatively charged molecules on rice cells may play an important role in these responses [19]. Chemical and physical properties of chitosan such as viscosity, acetylation degree, and molecular size should be taken into account in this interaction. Chitosan application has been shown

Table 4.1 Enzymes associated with the resistance induction in rice by chitosan

Enzymes	Chitosan type	Concentration	Biological system	References
PAL	Chitosan 85% DD★	0.20 mg/mL	3-week-old seedlings	[4]
PAL	Chitosan 85% DD	0.6 mg/L	3-week-old seedlings	[13]
Chitinase	Hydrolyzed chitosan (dp 3–9)	500 mg/L	18-day-old seedlings from chitosan-treated rice seeds	[27]
Chitosanase	Chitosan 63% DD	1000 mg/L	18-day-old seedlings from chitosan-treated rice seeds	[27]
β-1,3-Glycanase	Chitosan 63% DD	1000 mg/L	18-day-old seedlings from chitosan-treated rice seeds	[27]
Peroxidase	Chitosan 85% DD	0.20 mg/mL ¹	3-week-old seedlings	[4]
Polyphenol oxidase	Chitosan 85% DD	0.6 mg/L	3-week-old seedlings	[13]

★DD, degree of deacetylation.

to promote the biosynthesis of phytoalexins in rice [20]. In this regard, momilactones and oryzalexins are diterpenic rice phytoalexins, compounds that have a potent antimicrobial activity [29]. Flavonoid and diterpenic phytoalexins accumulate in chitosan-treated rice seedling leaves [30].

Some enzymes associated with the resistance induction by chitosan are listed in Table 4.1.

CHITOSAN'S PROTECTION OF RICE AGAINST DISEASES UNDER CONTROLLED CONDITIONS

Many rice diseases are transmitted by seed. The first effort to control rice diseases is to achieve a healthy seed. Several seed treatments are carried out, usually using expensive chemicals such as benomyl, tebuconazole, and their combinations. They are effective in controlling most of the fungal pathogen on the rice seed surface, but not in controlling bacteria and viruses [31]. However, they do not have a great effect on promoting germination and increasing the germination rate and seedling growth indexes.

Chitosan application on seeds by soaking and foliar spraying four times showed disease control and significantly increased rice yield [32]. Soaking the rice seeds with polymeric chitosan at concentrations ranging from 50 mg/L to 150 mg/L showed positive effects on rice seed storability, which was better than those obtained by soaking them in oligomeric chitosan and water [33]. Chitosan application also protects rice seeds against *Fusarium* sp. The biggest protection index achieved was 93.3% at 1000 mg/L at the ninth day [34]. Rice seeds treated with chitosan can also provide protection to the emerging seedlings; thus, 18-day-old seedlings from chitosan-treated rice seeds were highly resistant against *P. grisea* attack [27].

Fertilizer supplements, herbicide applications, and irrigation programs do not detrimentally influence chitosan seed applications [8]. Other commercial seed treatments (e.g., insecticides and fungicides) should be applied prior to chitosan. The components already present on the seed will be attached to them by chitosan, which forms a “cellophane-like” surface on the seed after drying.

Chitosan foliar spraying also protects plants against diseases. Chitosan application at 80 mg/L in combination with chemical fertilizer reduced infection of *B. oryzae* in both inoculated and noninoculated rice plants [35]. These authors also suggested that it might be possible to reduce seed infection by dirty panicle disease by spraying chitosan during the crop season.

A comparative experiment under greenhouse conditions showed a tendency to achieve higher rice yield when polymeric chitosan was applied by seed soaking before planting, followed by foliar spraying. Polymeric chitosan treatment tended to be better than oligomeric chitosan and monomer [36].

Chitosan foliar application significantly reduced bacterial leaf blight and leaf streak incidence and severity by comparison with the control under greenhouse conditions. Furthermore, chitosan solutions significantly increased the activities of PAL, peroxidase, and polyphenol oxidase in rice seedlings following inoculation of two bacterial pathogens. These chitosan solutions also inhibited bacterial growth. In conclusion, the role of chitosan in protecting rice against bacterial pathogens involves direct antibacterial activity and indirect activity by induction of resistance.

Application of chitosan solution in Vietnam fields significantly increased (~31%) rice yields. In that study, chitosan treatments were applied by spraying three times during a 120-day rice-growing period. Incidence of common brown backed rice plant hopper attack was reduced about 25, 30.5, and 35% in chitosan-treated plants at concentrations of 50, 70, and 100 ppm, respectively, compared to 63.2% in control plants [37]. Brown plant hopper

transmits viral diseases such as grassy stunt and ragged stunt. The plant hopper family also transmits the tungro and dwarf virus diseases. Therefore, if chitosan dramatically reduces plant hopper attacks, viral disease incidence is expected to decrease too. Chitosan successfully applied by Thai farmers has shown activity in the self-defense system of plants and as a growth promoter to increase agricultural production [38].

Chitosan application in Cuban rice fields improved yields and provided partial protection against *P. grisea*. Chitosan obtained from lobster shells, and applied at 1000 mg/L as a seed treatment achieved good levels of control of this fungus. Experiments were carried out in the field considering natural high infestation levels. Initially, significant differences were not found at the seedling stage between chitosan-treated and control treatments. However, by the second evaluation on rice panicles neck disease, chitosan-treated plants showed good resistance to *P. grisea* attack.

PROSPECTS AND PROBLEMS INVOLVED IN USING CHITOSAN TO PROTECT RICE

Chitosan protection of rice against major diseases is well documented, but still other important aspects should be considered in further studies, including the type of chitosan to be applied, the stability of the chitosan formulation, frequency and mode of application, and concentration. These aspects should be well known before carrying out direct applications of this compound on rice crops. Nowadays, the main studies on chitosan for agricultural purposes are focused on improving the stability of the chitosan formulation, increasing its antimicrobial action [39], improving the eliciting activity, and investigating its effects on gene regulation [25]. In this sense, application of the nanotechnology opens new and unexplored possibilities to foster new properties in chitosan derivatives [40]. However, the application of this new nanotechnology requires properly and scientifically assessing all possible side effects that may be associated with its practical use on living systems to avoid any undesirable effects [41].

CONCLUDING REMARKS

Chitosan has attracted the attention of many researchers worldwide due to its versatile properties as a natural biopolymer, its biocompatibility, and its various applications. Its use in plant protection, especially in rice crops, provides an attractive alternative to chemical control of pathogenic

microorganisms, which nowadays is a highly questioned method due to its toxicity in humans and the environment. Cuban researchers have extensively studied the use of chitosan to protect rice against its major diseases. So far, we have well established that chitosan acts directly against rice pathogenic fungi and bacteria, and that it induces defense mechanisms that further protect rice plants. We have reported that chitosan application reduces insect attack, which transmits viral diseases. Additionally, our results show that chitosan improves rice yield and enhances seed germination and plant growth.

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CHAPTER 5

Integrated Application of Chitosan Coating with Different Postharvest Treatments in the Control of Postharvest Decay and Maintenance of Overall Fruit Quality

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INTRODUCTION

Fruits and vegetables are important components of the human diet. Their quality attributes include skin color, fragrance, flavor, taste, and texture, which are linked with consumer acceptance. Fruits and vegetables undergo progressive deterioration after harvest due to respiration and other biochemical processes, desiccation, and microbial infection and growth. Postharvest decay increases during prolonged storage, often as a result of physiological changes that may allow the pathogens to infect and establish in the fruit [1]. Postharvest decay has been identified as one of the major cause for postharvest losses, and it was reported to be more than 40% in developing countries [2]. Storage temperature influences the decay, and decay and insect infestation are currently mainly controlled with synthetic chemicals. Due to global concern over the use of these chemicals and their often-hazardous side effects on the environment and human health, more stringent product registration requirements have been developed and applied. One approach to replacing chemical fungicides in controlling postharvest diseases includes chitosan coating of fruits. This chapter reviews the effectiveness of fruit coatings on the control of postharvest diseases and the effects of fruit coatings on the quality and chemistry of the fruit. Emphasis is particularly on ways in which the beneficial effects of chitosan may be improved and enhanced.

FRUIT COATINGS

Several fruit coatings have been used both experimentally and commercially for various purposes where the fruit are dipped or sprayed to improve their appearance or delay deterioration. Many natural waxes and coating derived from oil refining are used including FMC 705, FMC 214, and Nature Seal carnauba and polyethylene coatings especially on citrus fruits. Much of their effect is cosmetic, but coating kinnow fruits (*Citrus nobilis* x *Calamia deliciosa*) with commercial waxes (Fruitex, Britex561, or Seal Britex-56) was shown also to increase their shelf life by up to 60 days [3,4]. An experiment was conducted by dipping cranberry fruits in BioSave 110 (a commercial preparation containing the biocontrol agent *Pseudomonas syringae*) and coating them with carnauba wax that consistently resulted in lower disease incidence than noncoated fruits over a 16-week storage period [5]. Cassava roots (*Manihot esculenta*) deteriorate within a day or so after harvesting but a paraffin wax coating is currently applied to roots exported by ship from Costa Rica to Europe and the United States. To achieve this, they are cleaned thoroughly directly after harvest, placed in an oven at 50°C for 2 h, then immersed, while still hot, in paraffin wax at 62°C, cooled, and transported at 12°C [6]. Because little evidence shows that waxing controls diseases, chemical fungicides have in the past been commonly included in the wax. For example, limes (*Citrus aurantifolia*) coated with wax containing 0.1% of either thiabendazole or benomyl remained green and disease free and suitable for marketing after 3–4 weeks in storage [7].

Other coatings that have been applied to fruit and vegetables include Tal Prolong, which consisted of sucrose esters of fatty acids, and carboxymethyl cellulose, which could delay the ripening of bananas of *Musa* (AAA group “Kluai Hom Thong”) [8] and reduce levels of disease on stored apples [9]. Semperfresh has a similar composition, and coated bananas showed delayed development of crown rot caused by infection with *Colletotrichum musae*. However, when fruits were inoculated with *C. musae* after harvest the results were inconsistent [10]. The control of crown rot could be enhanced by the inclusion of organic acids to the coating material [11]. Lazan et al. [12] described another fruit coating, marketed as Vapor Gard, which was shown to reduce water loss, retard softening, reduce the decrease in ascorbic acid content, inhibit malic enzyme activity, and increase polygalacturonase activity compared to untreated fruit. Li and Tian [13] reported that coating fruit with trehalose (also called mycose or tremalose) and other sugars could help to retain the viability of the biocontrol yeast *Cryptococcus laurentii* in controlling postharvest diseases of apples (*Malus domestica*).

PROPERTIES OF CHITOSAN

Chitosan is derived from chitin and is a copolymer of β (1-4)-acetamido-D-glucose and β (1-4)-2-amino-2-deoxy-D-glucose units in varying proportions. It can be used to produce films for coating fruit and vegetables that are selectively permeable to gases such as CO₂, O₂, and ethylene [14,15]. Chitosan exhibits antimicrobial properties, but it must be dissolved in an acid solution to activate these properties. It also exhibits eliciting properties; that is, chitosan can draw out the fruit's or vegetable's natural resistance to microbial infection. The antifungal property of chitosan is mainly associated with the damage on the fungal plasma membrane [16] and is considered a promising alternative to synthetic fungicides [17,18]. Devlieghere et al. [19] stated the -NH₂ group of chitosan could be responsible for the prevention of proliferation of decay causing pathogens in foodstuff. Chitosan has been shown to reduce the growth of decay-causing fungi and induce resistance responses in host tissues as well as produce other effects that contribute to reduced decay [20]. Chitosan has been successfully used to control various postharvest diseases including brown rot (caused by *Monilinia fructicola*) on peaches (*Prunus persica*) [21] and nectarines [22] and gray mold in inoculated grapes [23] and reduce decay of blueberry (*Vaccinium corymbosum*) during storage [24]. Chitosan has other properties and has been shown to improve the postharvest life of apples [15], mangosteen (*Garcinia mangostana*) [25] and strawberries (*Fragaria x ananassa*) [26], Japanese pears (*Pyrus ussuriensis*) and kiwifruit (*Actinidia deliciosa*) [27], peppers (*Capsicum annuum*) and cucumbers (*Cucumis sativus*) [28], longans (*Euphoria longana*) [29], oyster mushrooms (*Pleurotus ostreatus*) [30], breadfruit (*Artocarpus altilis*) [31], guava (*Psidium guajava*) [32], papaya (*Carica papaya*) [33], and broccoli (*Brassica oleracea*) [34]. Conversely chitosan was shown to have no positive effects on papaya [35] and, in contrast to the previously mentioned study, strawberries [36]. In bananas (*Musa* AAA group "Kluai Hom Thong"), chitosan coating significantly delayed ripening as in terms of peel color, firmness, total soluble solids, and disease severity [37]. Apart from the examples already mentioned, chitosan also was shown to improve fruit quality (total soluble solids, firmness, anthocyanin concentration) in strawberries at 4°C [28]. Zhang and Quantick [38] stated that chitosan coating delayed the changes in the contents of anthocyanins, and total phenol contents in litchi (*Litchi chinensis*). It was also shown to reduce the activities of the browning enzymes such as polyphenol oxidase (PPO) and peroxidase (POD). Being biodegradable and eco-friendly, chitosan films are useful as an alternative

to synthetic packaging films. Chitosan films exhibit excellent gas barrier properties for food packaging and can be produced by solution casting methods. Srinivasa et al. [39] found that nonwrapped mango fruits stored in cartons had a postharvest life of 8–10 days at $27 \pm 1^\circ\text{C}$ at 65% RH. Fruits stored in the same conditions in cartons whose top surface was covered in chitosan film could be stored for up to 18 days without any microbial growth and off-flavors.

Chitosan is commonly applied as a dip or spray to fruit and vegetables directly after harvest; however, application has also been tested while the fruit are still on the plants. Tezotto-Uliana et al. [40] found that when chitosan was applied to raspberries at 2% preharvest, they retained key quality attributes for 12 days, while when it was applied at 1 or 2% postharvest they retained key quality attributes for 15 days.

CHITOSAN IN COMBINATION WITH MODIFIED ATMOSPHERE PACKAGING

Modified atmosphere packaging is where the fruit or vegetable is enclosed within sealed plastic film, which is slowly permeable to the respiratory gases. The gases will change within the package, thus producing lower concentrations of O_2 and higher concentrations of CO_2 than exists in fresh air. While modified atmosphere packaging preserves fruits and vegetables, especially combined with reduced temperatures, it has been shown to be enhanced by first coating the fruit before packaging. Coating the fruit with chitosan before sealing them in biorientated polypropylene film reduced the enzymatic browning of the litchi pericarp and controlled fruit decay [41]. However, there were differences in cultivar responses where the combined treatment completely prevented the pericarp browning in cv. McLean's Red, whereas cv. Mauritius still showed incidences of browning; also Mauritius had a higher respiration rate than McLean's Red during storage. This limited the combination treatment because presorting is not possible for MAP packaged fruits. The loss of anthocyanin and the browning index increased with the PPO and POD activity. Zhang and Quantick [38] also showed chitosan coating inhibited PPO and POD activity and retained the anthocyanin content in the pericarp of the litchi cv. Huaizhi for up to 14–15 days, and the oxidative enzyme activity increased after 21 days in storage. De Reuck et al. [41] confirmed that the combination of chitosan and MAP significantly decreased the PPO and POD activities in both McLean's Red and Mauritius and the inhibitory effect on the PPO and POD activities was higher in McLean's Red

than in Mauritius. The chitosan and MAP treatment also reduced the electrolyte leakage from the pericarp and maintained the membrane integrity. The loss of integrity of cell membranes is due to malfunction of membrane lipid biosynthesis and membrane repair due to shortage of adenosine triphosphate, causing ion leakage and cellular decompartmentalization. Consequently, the browning reactions take place when the anthocyanins come into contact with the oxidizing enzymes PPO and POD. Moreover, the combined chitosan and MAP treatment reduced the rate of respiration significantly in McLean's Red, and the soluble solids concentration and the titratable acidity were higher in McLean's Red packaged in MAP [41].

Foodborne pathogens that may be of concern with respect to litchi are *Escherichia coli* O157:H7 and *Staphylococcus aureus*. The presence of *E. coli* O157:H7 and *S. aureus* on fruit are indicators of fecal contamination and poor hygiene, respectively. Fruit stored at low temperatures may limit the range of pathogens that can grow on fruit surfaces. Fruit kept at temperatures below 7–8°C will allow for the survival of the foodborne pathogens. Moreover, incorrect handling of fruit, leading to mechanical damage, will cause breaks in the protective epidermal barrier, which limits shelf life of the fruit and provide more entry points for postharvest and foodborne pathogens. With respect to integrated (MAP and 1 g/L chitosan) treatments, the total microbial population was significantly lower compared to the other treatments maintained at both temperatures (Table 5.1). The integrated treatment (MAP and 1 g/L chitosan) significantly reduced the total yeast population after 2 days at 20°C. Our investigation suggests that yeasts tend

Table 5.1 Microbial counts (log CFU/cm²) of litchi integrated with MAP and chitosan, and MAP and 1-MCP after cold storage

Treatment	Storage days	Total bacterial count	Total fungal count	Total yeast count
Control	Immediate	2.98 ± 0.54 ^a	2.63 ± 0.07 ^a	2.80 ± 0.21 ^{b,c}
	21 days at 2°C	2.64 ± 0.31 ^a	1.98 ± 0.35 ^b	2.59 ± 0.05 ^{d,c,e}
	2 days at 20°C	3.35 ± 0.59 ^a	1.99 ± 0.09 ^{a,b}	2.66 ± 0.07 ^{d,c}
MAP + 0.1 g/L chitosan	Immediate	2.98 ± 0.54 ^a	2.63 ± 0.07 ^a	2.80 ± 0.21 ^{d,c}
	21 days at 2°C	3.60 ± 0.09 ^a	1.51 ± 0.28 ^c	2.42 ± 0.58 ^{d,e}
	2 days at 20°C	3.61 ± 0.24 ^a	1.99 ± 0.56 ^b	3.83 ± 0.02 ^a
MAP + 1.0 g/L chitosan	Immediate	2.98 ± 0.54 ^a	2.63 ± 0.07 ^a	2.80 ± 0.21 ^{b,c}
	21 days at 2°C	1.66 ± 0.87 ^b	0.62 ± 0.45 ^d	2.08 ± 0.82 ^e
	2 days at 20°C	1.12 ± 0.37 ^b	0.92 ± 0.21 ^d	1.00 ± 0.31 ^f

Means followed by the same letter do not differ significantly at the 0.05% level of the Fisher's Protected Least Significant Test.

Means in the same column with different letters are significantly different ($P < 0.05$).

to be more sensitive to the antimicrobial action of chitosan than bacteria, which correlates with our observations. However, the total yeast and fungal population in integrated treatment (MAP and 0.1 g/L chitosan) was observed to increase significantly when fruit were kept at 20°C.

Enumeration of viable cells showed a significant decrease in both high and low inoculum loads of *E. coli* O157:H7 and *S. aureus* in all treatments after 21 days of cold storage (Table 5.2). The antimicrobial activity of chitosan treatment significantly reduced the high *E. coli* O157:H7 and *S. aureus* loads within 2 h before processing commenced. The level of high inoculum load was significantly reduced by both chitosan treatments. Chitosan 0.1 g/L reduced *E. coli* O157:H7 loads to low levels after 21 days of cold storage. However, *E. coli* O157:H7 survived and increased by 0.75 logs after fruit were kept at 25°C for 2 days. Chitosan 1 g/L also significantly decreased *E. coli* O157:H7 counts with 2.5 logs. A significantly lower *E. coli* O157:H7 counts were observed in the 1 g/L chitosan treatment compared to the

Table 5.2 Effect of integrated postharvest treatments on food-borne pathogen survival (log CFU/cm⁻²) on litchi fruit surfaces

	Control	MAP + chitosan 0.1 g/L	MAP + chitosan 1.0 g/L
<i>E. coli</i> O157:H7 (high inoculum load)			
Immediate	4.30 ± 0.31 ^a	1.81 ± 0.44 ^{b,c,d}	0.50 ± 0.12 ^{f,g}
21 days at 2°C	0.95 ± 0.56 ^{e,f}	1.53 ± 0.24 ^{c,d}	0.31 ± 0.28 ^g
2 days at 20°C	1.90 ± 0.19 ^{b,c}	0.09 ± 0.00 ^g	0.01 ± 0.00 ^g
<i>E. coli</i> O157:H7 (low inoculum load)			
Immediate	2.33 ± 0.22 ^a	1.90 ± 0.03 ^{b,c}	0.092 ± 0.00 ^h
21 days at 2°C	0.28 ± 0.19 ^{g,h}	0.01 ± 0.00 ^h	0.01 ± 0.00 ^h
2 days at 20°C	1.47 ± 0.23 ^{c,d}	0.34 ± 0.00 ^{g,h}	0.08 ± 0.02 ^h
<i>S. aureus</i> (high inoculum load)			
Immediate	4.24 ± 0.10 ^a	3.47 ± 0.23 ^{b,c}	3.19 ± 0.47 ^c
21 days at 2°C	2.34 ± 0.14 ^d	1.09 ± 0.56 ^{f,g}	0.01 ± 0.00 ^g
2 days at 20°C	1.60 ± 0.52 ^{e,f}	0.86 ± 0.6 ^g	0.67 ± 0.28 ^g
<i>S. aureus</i> (low inoculum load)			
Immediate	2.06 ± 0.20 ^a	2.16 ± 0.49 ^a	0.27 ± 0.37 ^d
21 days at 2°C	0.39 ± 0.08 ^{c,d}	0.01 ± 0.12 ^d	0.01 ± 0.00 ^d
2 days at 20°C	1.38 ± 0.20 ^b	0.01 ± 0.05 ^d	0.30 ± 0.00 ^d

Means followed by the same letter do not differ significantly at the 0.05% level of the Fisher's Protected Least

Significant Test. High inoculation load (1×10^6 CFU/mL). Low inoculation load (1×10^4 CFU/mL).

Means in the same column with different letters are significantly different ($P < 0.05$).

0.1 g/L chitosan treatment after 21 days of cold storage. After fruit were removed from cold storage, fruit were kept at room temperature, which did not promote any significant growth or inhibition. Low inoculum loads of *E. coli* O157:H7 and *S. aureus* were used in our investigation to simulate a more realistic scenario that may potentially occur during supply chain operations. Foodborne pathogen counts significantly decreased in all treatments when fruit were kept at 2°C for 21 days. However, removing fruit from cold storage resulted in increased pathogenic bacterial counts. Litchi fruit treated with chitosan 0.1 g/L significantly reduced *E. coli* counts to the extent that no bacterial colonies were found. Chitosan 1 g/L immediately reduced low inoculum loads of *E. coli* O157:H7 counts (1.5 log). Following cold storage, few cells were enumerated and higher counts were observed when fruit were kept at 20°C. Chitosan treatment significantly inhibited *S. aureus*, and after 2 days at room temperature, lower bacterial counts were obtained. Thus, chitosan 1 g/L in combination with MAP had the best inhibitive effect on *E. coli* O157:H7 and *S. aureus*.

The effect of integrated modified atmosphere packaging and postharvest treatments on *Penicillium chrysogenum*, *Penicillium crustosum*, *Penicillium expansum*, *Penicillium glabrum*, and *Penicillium solitum* decay are given in Table 5.3. No disease was recorded on fruit treated with MAP and chitosan (1 g/L) treatments. Fruit decay incidence due to *P. chrysogenum* and *P. glabrum* was completely controlled in combination treatment with MAP and chitosan at 0.1 or 1.0 g/L concentrations during low temperature storage for 21 days at 2°C. On the other hand, combination treatment with MAP and chitosan 1 g/L effectively controlled the fruit decay incidence by *P. crustosum* and *P. expansum* under the previously mentioned storage condition. The direct fungal activity of chitosan against *Penicillium* spp. is dependent on its concentration. The application of the integrated MAP and chitosan dip treatments provided an alternative control of decaying and foodborne pathogens. As means to ensure safe quality fruit, an integrated approach is needed to successfully adopt integrated MAP and postharvest technology.

Leceta et al. [42] showed that MAP-packaged ready-to-eat baby carrots (*Daucus carota*) that had previously been coated with chitosan had delayed microbial spoilage without causing adverse impacts on their quality. The microorganisms they observed in this study were *Bacillus cereus*, total coliforms, *Pseudomonas* spp., *S. aureus*, total viable counts, yeast, and molds. Sensory analysis showed that overall acceptability of coated baby carrots was similar to uncoated samples. Strawberries were treated with a solution of 1% chitosan, packaged in MAP film packages with either 80% O₂ or 5% O₂, with the balance nitrogen, and then stored at 4, 8, 12, or 15°C. The coating inhibited

Table 5.3 Effect of integrated modified atmosphere packaging and postharvest treatment on *Penicillium* spp. decay after 21 days at 2°C

Treatment	Disease incidence (%)	Lesion diameter (mm)
<i>P. chrysogenum</i>		
Control	86.67	23.17 ± 2.01 ^a
MAP and 300 nL/L 1-MCP	93.33	23.57 ± 1.78 ^a
MAP and 1000 nL/L 1-MCP	100	23.98 ± 2.85 ^a
MAP and 0.1 g/L chitosan	0	0 ± 0 ^b
MAP and 1.0 g/L chitosan	0	0 ± 0 ^b
<i>P. crustosum</i>		
Control	100	23.014 ± 2.60 ^a
MAP and 300 nL/L 1-MCP	96.67	21.732 ± 4.62 ^a
MAP and 1000 nL/L 1-MCP	93.33	33.392 ± 1.83 ^b
MAP and 0.1 g/L chitosan	3.33	1.75 ± 3.03 ^c
MAP and 1.0 g/L chitosan	0	0 ± 0 ^c
<i>P. expansum</i>		
Control	100	29.22 ± 2.27 ^a
MAP and 300 nL/L 1-MCP	100	32.983 ± 1.42 ^{a,b}
MAP and 1000 nL/L 1-MCP	100	30.65 ± 1.91 ^b
MAP and 0.1 g/L chitosan	3.33	0.58 ± 1.01 ^c
MAP and 1.0 g/L chitosan	0	0 ± 0 ^c
<i>P. glabrum</i>		
Control	76.67	22.53 ± 2.50 ^a
MAP and 300 nL/L 1-MCP	76.67	19.18 ± 5.78 ^a
MAP and 1000 nL/L 1-MCP	90	29.1 ± 4.06 ^b
MAP and 0.1 g/L chitosan	0	0 ± 0 ^c
MAP and 1.0 g/L chitosan	0	0 ± 0 ^c
<i>P. solitum</i>		
Control	100	22.53 ± 2.50 ^a
MAP and 300 nL/L 1-MCP	100	29.65 ± 2.87 ^b
MAP and 1000 nL/L 1-MCP	100	27.72 ± 1.53 ^b
MAP and 0.1 g/L chitosan	3.33	0.33 ± 0.58 ^c
MAP and 1.0 g/L chitosan	0	0 ± 0 ^c

Means followed by the same letter do not differ significantly at the 0.05% level of the Fisher's Protected Least Significant Test.

Means in the same column with different letters are significantly different ($P < 0.05$).

the growth of microorganisms at all temperatures, especially when combined with high O₂ that also seemed to help in retaining their color [43].

The interaction between chitosan coating, MAP packaging, and intermittent warming has also been evaluated. Physiological disorders, such

as browning of the pulp, chilling injury, and fruit softening, affect the marketability of fruit. An example would be where postharvest losses in peaches due to the pulp browning was related to the activity of PPO acting on the substrate, due to cell wall deterioration that resulted from chilling injury. Generally, enzymatic browning in fresh fruits can be controlled by the application of sulfites, but the food regulations imposed by the U.S. Food and Drug Administration do not allow the application of sulfites on fresh produce. However, Ruoyi et al. [44] showed that application of 1% chitosan + 0.5% calcium chloride + MAP package + intermittent warming treatment maintained higher ascorbic acid content by reducing the chilling sensitivity of the fruit and softening by inhibiting the ascorbic acid oxidases and polygalacturonase.

CHITOSAN IN COMBINATION WITH PHYSICAL TREATMENTS

Chitosan and Heat Treatment

Apples cv. Gala were exposed to 38°C for 4 days before or after being coated with 1% chitosan and then stored at 0°C for 8 weeks followed by 20°C for 7 days as shelf life. The heat treatment plus chitosan reduced their respiration rate, ethylene production, malondialdehyde (MDA), and membrane leakage, and resulted in greater firmness and consumer acceptance than those not treated. However, some negative effects were observed in regard to fruit color, titratable acidity, and weight loss [45].

Chitosan and Hypobaric Storage

Romanazzi et al. [46] found that the combination of spraying sweet cherries with chitosan at 0.1, 0.5 and 1.0% 7 days before harvest and storage under 0.5 atmospheres for 4 h directly after harvest effectively controlled fungal decay during 14 days storage at $0 \pm 1^\circ\text{C}$, followed by a 7-day shelf life. Fungi associated with rots included brown rot, gray mold (*Botrytis cinerea*), blue mold (*P. expansum*), Alternaria rot, (*Alternaria alternata*), and Rhizopus rot (*Rhizopus stolonifer*).

CHITOSAN IN COMBINATION WITH PLANT DERIVATIVES

Chitosan and Essential Oils

Avocado fruit are commonly dipped in the synthetic nonsystemic fungicide prochloraz in the packing line to control anthracnose disease caused by *Colletotrichum gloeosporioides*, which is a common commercial packinghouse treatment adopted in many avocado-exporting countries. Bosse et al. [47] reported that the postharvest losses due to anthracnose can be as high as

80% if the fruits are not treated with prochloraz as soon as possible after harvesting. But, as indicated earlier, the demand for a reduction in the use of postharvest chemicals continues to grow. In addition, commercial wax formulations including Avoshine® canuba wax is used for avocados [48], but some consumer resistance to waxing of fruits, including avocados, has been observed in the European Union (EU). Several countries, including South Africa, Israel, and Chile, export large amount of avocados to the EU and anthracnose affects their quality, marketability, and shelf life [49].

Thyme oil has been shown to have antifungal activity against *C. gloeosporioides* both *in vitro* and *in vivo* in the avocado cvs. Hass and Fuerte [50]. Integrated application of chitosan coating (1%) and thyme oil (1%) at 1:1 (v/v) and 3:1 (v/v) concentrations, improved its antifungal activity against *C. gloeosporioides in vitro*. Also, the combined treatment at both concentrations 1:1 and 3:1 showed fungicidal activity whereas, chitosan coating alone showed fungistatic activity against this fungus. During *in vivo* investigations in cv. Hass with chitosan (1%) and thyme oil (1%) [3:1 v/v], anthracnose incidence and severity were significantly reduced (Figure 5.1) during preventive and curative applications [51]. The mode of action of combined application showed that fruits infected with *C. gloeosporioides* and

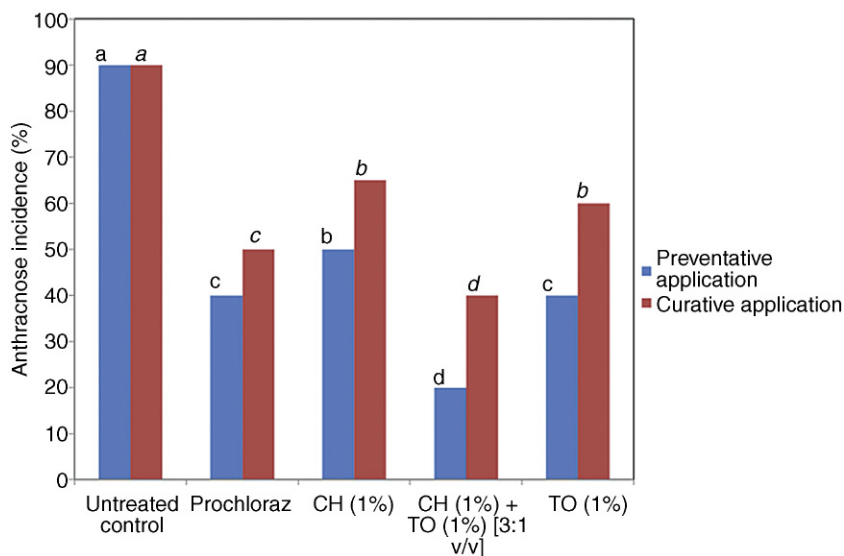


Figure 5.1 Effect of Chitosan and Thyme Oil Treatment on the Control of Anthracnose in Avocado cv. Hass. The means in each bar with the same letter are not significantly different, $P < 0.05$. TO, thyme oil; CH, chitosan. Above each column means followed by a common letter are not significantly different ($P < 0.05$).

coated with chitosan and thyme oil stimulated significantly the induced defense enzymes phenylalanine ammonia-lyase (PAL), POD, chitinase, β -1,3-glucanase, and the antioxidant enzymes superoxide dismutase (SOD) and catalase (CAT) than the prochloraz fungicide treatment (Figure 5.2A–D, Figure 5.3A–B). This indicated that the mode of action of the chitosan and thyme oil integrated treatment was due to the enhanced activities of defense

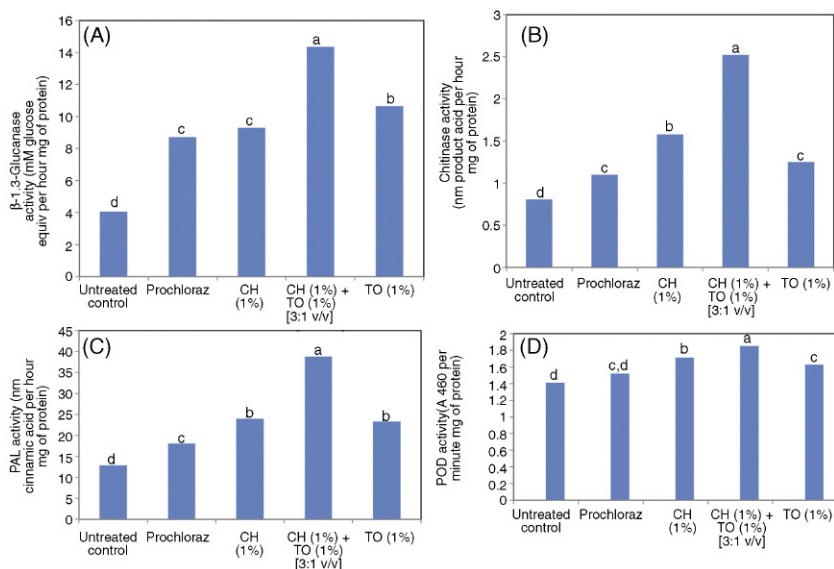


Figure 5.2 Effect of Chitosan and Thyme Oil Treatment on Defense Enzyme Activities. (A) 1-3 β glucanase; (B) chitinase; (C) PAL; (D) POD activities in avocado cv. Hass. The means in each bar with the same letter are not significantly different, $P < 0.05$. TO, thyme oil; CH, chitosan. Above each column means followed by a common letter are not significantly different ($P < 0.05$).

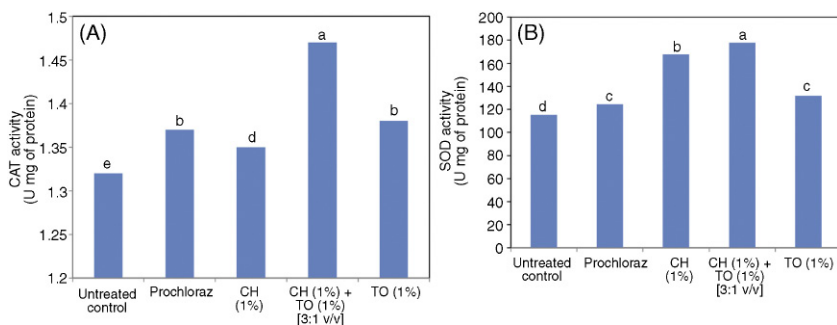


Figure 5.3 Effect of Chitosan and Thyme Oil in Combination Treatment on Antioxidant Enzymes. (A) CAT activity; (B) SOD activity in avocado cv. Hass. The means in each bar with the same letter are not significantly different, $P < 0.05$. TO, thyme oil; CH, chitosan. Above each column means followed by a common letter are not significantly different ($P < 0.05$).

response-related enzymes [51]. The integrated application of chitosan and thyme oil significantly enhanced the production of total phenolic content and the antioxidant activity in infected avocado fruits (Figure 5.4A–B). The phenolic rings and hydroxyl groups on the phenol rings of thymol and cymol (active volatile compounds) are responsible for the enhanced antimicrobial activity of the thyme oil [52]. The combined effect of essential oils and chitosan coating was shown to improve the chitosan coating's antifungal properties [36,37,53]. Bill et al. [51] showed an increase of total phenolic compounds in fruits coated with chitosan incorporated with thyme oil. Anthracnose decay control by chitosan coating was reported in climacteric fruits during postharvest storage including papaya (*C. gloeosporioides*) [54,55], mangoes (*C. gloeosporioides*) [56], and bananas (*C. musae*) [57].

PAL is the primary enzyme involved in the biosynthesis of phenols [58]. Romanazzi et al. [59] showed that chitosan coating induced PAL enzyme activity. However, incorporation of thyme oil into chitosan coating helped to improve the synthesis of phenolic compounds by significantly inducing PAL activity. Fajardo et al. [60] and Zhang and Quantick [38] showed that chitosan coating improved the chitinase and β -1,3 glucanase activities in oranges, strawberries, and raspberries. Incorporation of thyme oil into chitosan coating improved the activities of chitinase, β -1,3-glucanase, and POD in avocado fruit. Chitinase and β -1,3-glucanase play a major role in the plant defense mechanisms against fungal pathogens by facilitating the biochemical reactions involved in hydrolyzing polymers in fungal cell walls [61,62]. POD

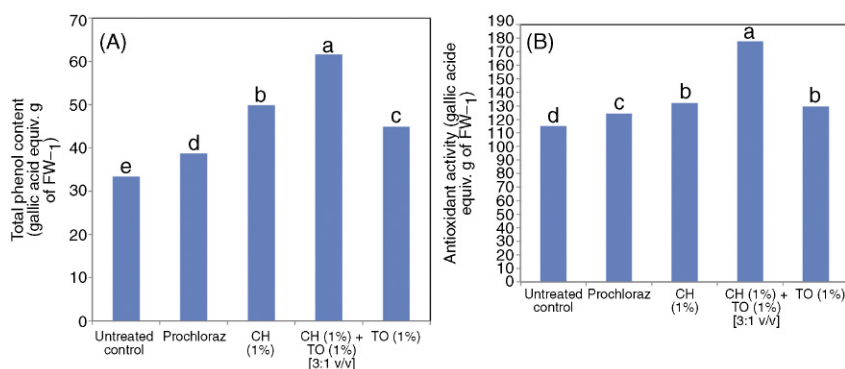


Figure 5.4 Effect of Chitosan and Thyme Oil Combination Treatment for Phenol Content and Antioxidant Activity. (A) total phenol content; (B) antioxidant activity in avocado cv. Hass. The means in each bar with the same letter are not significantly different, $P < 0.05$. TO, thyme oil; CH, chitosan; FW, fresh weight. Above each column means followed by a common letter are not significantly different ($P < 0.05$).

was linked with disease resistance and involved in production of phenolic cross-links connecting adjoining biopolymer chains [63]. Similar observations were reported with the integrated application of chitosan coating and methyl jasmonate on cherry tomatoes infected with *A. alternata* [64] and peaches (cv. Baifeng) [65]. The antioxidant enzymes were reported to show a positive relationship with plant resistance to pathogens. Reactive oxygen species (ROS), O_2 , and peroxide (H_2O_2) were reported to be involved during the early stage of defense mechanisms that correlated with plant resistance to pathogens [66]. However, Baker and Orlandi [67] found that during latter stages of pathogenesis, the increased production of ROS can contribute to cell degeneration and SOD, mediates the dismutation reaction of O_2 into H_2O_2 , and the POD and CAT convert H_2O_2 to oxygen and water. The integrated application of chitosan coating and thyme oil increased antioxidant enzyme activities (SOD, POD, and CAT) in the avocado fruit (cv. Hass), prevented the degeneration of infected cells by *C. gloeosporioides*, protected the cell membrane structure and function of the fruit tissue, and maintained higher levels of phenolic content (antioxidant capacity). All these biochemical changes contributed to enhance the resistance of fruit tissue against invasion of *C. gloeosporioides* and to slow the spread of anthracnose. Fruit texture plays a major role in consumer acceptance, and the chitosan coating resulted in the maintenance of fruit firmness by inhibiting the activities of polygalacturonase, pectate lyase, and cellulase [68,69]. However, thyme oil incorporated in the chitosan coating reduced the incidence of anthracnose and thereby further maintained the fruit firmness and delayed ripening. The reduced ripening events were associated with delayed pulp color development (yellow, higher h^o). Similar findings were reported in oranges, where the integrated application of chitosan with either bergamot oil or tea tree oils delayed the fruit ripening and consequently the loss of firmness [70].

The combination of chitosan and thyme oil has also been shown to improve the postharvest quality and shelf life of shiitake (*Lentinus edodes*) mushrooms [71]. Coating shiitake with chitosan plus thyme oil maintained tissue firmness, inhibited the increase in respiration rate, and reduced micro-organism counts, such as yeasts, molds, and pseudomonas, during storage at $4 \pm 1^\circ C$ for 16 days. Also, coated shiitake had higher levels of total phenolics and flavonoids compared to the control treatment. The efficiency was better than that of thyme oil or chitosan coating separately.

Cinnamon oil contains cinnamaldehyde, β -caryophyllene, linalool, and other terpenes, compounds that are categorized as “generally recognized as safe” (GRAS) compounds. Li et al. [72] reported that sweet pepper

(*C. annuum* L.) had a short shelf life and the marketability was limited due to the incidence of fungal diseases. Combined application of chitosan coating and cinnamon oil showed higher SOD, POD, and CAT activities and MDA content, and electrolyte leakage was reduced during storage of 35 days [72]. They concluded that chitosan cinnamon oil coating might be a promising candidate for enhancing the keeping quality of sweet peppers. Lime fruit are commonly infected with sour rot caused by *Geotrichum candidum*. Currently, the synthetic fungicide Quazatine® is used at the packinghouses to control rots, but citrus essential oils and their constituents have shown to have fungicidal activities against these postharvest pathogens of citrus. Combination treatment of Citral 4 mL/L + chitosan 8 g/L gave almost 90% sour rot reduction in stored limes [73]. These applications of thyme oil and cinnamon oil showed synergistic effect during combination with chitosan coating on postharvest decay control.

Shao et al. [74] found that a coating of 1% chitosan alone effectively contributed to the control of green mold caused by *Penicillium digitatum* on citrus fruit. However, the synergistic antifungal activity of 1% chitosan coating combined with clove oil at 0.5, 1, or 2 mL/L was improved in *in vitro* studies on this fungus, but this effectiveness in controlling green mold in *in vivo* tests was not observed.

Perdones et al. [36] prepared film-forming dispersions with 1% high molecular weight chitosan and 3% lemon essential oil. These were used to coat strawberries (cv. Camarosa), which were then stored at 5°C. Chitosan coatings did not show a significant effect on acidity and soluble solid content of strawberries throughout storage; however, their respiration rate was slower and their antifungal activity higher, both in *in vitro* and *in vivo*, when inoculated with *B. cinerea*.

Chitosan and Plant Extracts

The effects of incorporating different concentrations of blueberry fruit and leaf extracts (BLE) into chitosan coating on the quality of fresh fruit during storage at $2 \pm 1^\circ\text{C}$ and $95 \pm 2\%$ RH for 35 days and then at room conditions for 3 days were investigated by Yang et al. [75]. They included five treatments such as 2% (w/v) chitosan coating (T1-Treatment 1), 2% (w/v) chitosan coating containing 4% (w/v) (T2), 8% (w/v) (T3), or 12% (w/v) (T4) BLE, and 2% (w/v) chitosan coating containing 12% BLE plus modified atmosphere packaging (giving an atmosphere of $3\text{ kPa O}_2 + 12\text{ kPa CO}_2$) (T5), and a control dipped into distilled water. The 2% chitosan coating that incorporated 8 or 12% BLE showed some degree of decreasing

decay rate on fruit compared with the control, and the coating with blueberry fruit and leaf extracts plus MAP had more effective control of fruit decay. This study suggested that chitosan coating incorporating BLE can be employed to extend shelf life and maintain high nutritional value of fresh blueberries during postharvest storage.

Chitosan and grapefruit seed extract treatments, alone or combined, significantly reduced postharvest fungal rot caused by *B. cinerea* of grapes (*Vitis vinifera* cv. Red globe) compared with controls. The combination also delayed rachis browning and dehydration, and maintained their visual appearance without detrimental effects on taste and flavor [76].

Chitosan and Plant Gums

Maqbool et al. [77] applied a composite coating of 10% gum arabic plus 1% chitosan to bananas postharvest. They showed that after 33 days of storage, coated fruit had 24% lower weight loss and 54% lower total soluble solids than those not coated. Fruit were firmer and had higher total carbohydrates and reduced sugars than those not coated.

CHITOSAN IN COMBINATION WITH ORGANIC POLYMERS

Arnon et al. [78] tested the efficacy of a newly developed polysaccharide-based edible bilayer coating comprising carboxymethyl cellulose (CMC) and chitosan in preserving postharvest quality of various citrus fruit, including “Or” and “Mor” mandarins, “Navel” oranges, and “Star Ru” by grapefruit, after simulated storage and marketing. In all citrus species, it was found that the CMC/chitosan bilayer coating was equally effective as the commercial polyethylene wax in enhancing fruit gloss. Furthermore, the CMC/chitosan bilayer coating slightly increased fruit firmness, especially of oranges and grapefruit, but was mostly not effective in preventing poststorage weight loss. Sensory analyses revealed that neither the CMC/chitosan bilayer coating nor the commercial wax coating had any deleterious effects on flavor preference of Navel orange and Star Ruby grapefruit. However, application of the commercial wax, and moreover the CMC/chitosan bilayer coating, resulted in a gradual decrease in flavor acceptability of Or and Mor mandarins because of increased perception of off-flavors. Flavor quality was slightly impaired in mandarins but not in oranges and grapefruit. Gol et al. [79] tested coating strawberries with carboxymethyl cellulose, hydroxypropyl methylcellulose and chitosan, alone and in combination, with an untreated control on their shelf life and overall quality during storage at

$11 \pm 1^\circ\text{C}$ and 70–75% RH. They found that coated fruit showed significant delays in weight loss, decay percentage, titratable acidity, total soluble solids, ascorbic acid content, and maintaining higher concentrations of total phenolics and total anthocyanins compared to noncoated control fruit.

Poverenov et al. [80] stored red bell peppers coated with chitosan, gelatin, or both chitosan and gelatin. The chitosan coating inhibited microbial spoilage and prolonged their storage, but the gelatin coating reduced softening but did not prolonged storage. However, the combination of both chitosan and gelatin coating was associated with a twofold decrease in microbial decay, reduced softening, and prolonged cold storage up to 21 days and fruit shelf life up to 14 days, without affecting their respiration rate or nutritional content.

Sánchez-González et al. [81] found that chitosan in combination with bergamot essential oil in grapes resulted in smaller weight losses and a greater antimicrobial effect, but it led to browner samples. Chitosan coatings containing bergamot oil were more effective than pure chitosan and hydroxypropyl methylcellulose coatings at reducing respiration rates. Of the coatings they tested, the most effective for Muscatel table grapes was chitosan containing bergamot oil because it showed the highest antimicrobial activity and the greatest control of respiration rates with a reasonably good control of water loss during storage. Gol et al. [79] tested coating strawberries with carboxymethyl cellulose, hydroxypropyl methylcellulose, and chitosan, alone and in combination, with an untreated control on their shelf life and overall quality during storage at $11 \pm 1^\circ\text{C}$ and 70–75% RH. They found that coated fruit showed significant delays in weight loss, decay percentage, titratable acidity, total soluble solids, ascorbic acid content while maintaining higher concentrations of total phenolics and total anthocyanins compared to noncoated control fruit.

CHITOSAN IN COMBINATION WITH ORGANIC SALTS

Anthrachnose disease is a major postharvest disease of papaya. Integrated applications with paraffin wax and ammonium carbonate $(\text{NH}_4)_2\text{CO}_3$ or sodium bicarbonate (NaHCO_3) formulations have decreased anthracnose in this fruit [82,83]. Infected fruits showed that $(\text{NH}_4)_2\text{CO}_3$ with chitosan completely inhibited disease incidence [84]. Also, the combined application of chitosan and $(\text{NH}_4)_2\text{CO}_3$ showed only 15% anthracnose incidence in naturally infected papaya fruit after 14-day low-temperature storage and 2 days at 13.5°C under simulated market conditions, while in the untreated

control fruit disease incidence was 80%. The recovery of *C. gloeosporioides* from the inoculated fruits after the combined application of chitosan and $(\text{NH}_4)_2\text{CO}_3$ was reduced to Log_{10} CFU/mL, while the untreated fruit and the chitosan and sodium bicarbonate combination application showed 2.01 and 7.89 Log_{10} CFU/mL, respectively. Anthracnose incidence of 30% was reported for fruit treated with $(\text{NH}_4)_2\text{CO}_3$ or chitosan alone and in fruit subjected to a combined treatment with NaHCO_3 and chitosan. $(\text{NH}_4)_2\text{CO}_3$ was reported to inhibit the mycelial growth and germination of *C. gloeosporioides* more effectively than sodium bicarbonate [82]. Therefore, application of chitosan with $(\text{NH}_4)_2\text{CO}_3$ or NaHCO_3 probably has a synergistic effect in the control of this disease. The effect is probably greater with $(\text{NH}_4)_2\text{CO}_3$ due to its higher inhibitory activity [82]. A chitosan and $(\text{NH}_4)_2\text{CO}_3$ combined application improved the overall fruit quality in papaya and improved skin and flesh color, eating quality after ripening, and also reduced weight loss during storage [84]. The overall marketable quality of these fruit after ripening was 70%. Untreated control fruits ripened faster, and the skin and flesh color development were significantly higher with higher hue angles (h° approx. 90; i.e., orange-yellow). Likewise, ripening of fruit and color development was delayed, and the skin color remained yellowish-green (h° approx. 70) while the flesh showed a h° angle of 60. Integrated application of $(\text{NH}_4)_2\text{CO}_3$ and chitosan also showed higher internal CO_2 compositions in the fruit cavity. In fruits treated with $(\text{NH}_4)_2\text{CO}_3$ and chitosan, the internal CO_2 concentrations increased up to 8% 2 h after treatment, then decreased to 7% and remained at 7% over the 14-day low temperature storage and 2-day market simulation conditions [84]. Whereas in untreated fruit and in those subjected to chitosan alone or in combination with NaHCO_3 , the internal CO_2 concentration was about 3–4%. The delayed color development, ripening, and increase in internal CO_2 concentration and fruit firmness and reduced weight loss, could be attributed to the semi-permeable and filmogenic properties of chitosan [28,85,86]. The presence of a chitosan coating with $(\text{NH}_4)_2\text{CO}_3$ on the fruit surface can prevent fruit-to-fruit disease transmission by acting as a physical barrier. This formulation could therefore be recommended to protect freshly harvested papaya and to reduce the postharvest losses, especially for long-term sea shipment, at least to destinations within 14-day from the harvest site.

Strawberries are a highly perishable nonclimacteric fruit, with a postharvest life that is affected by gray mold decay. During postharvest handling, these fruit are highly susceptible to moisture loss and mechanical injuries such as bruising. Calcium is a major component of the plant cell, and

increasing Ca content in the cell wall can provide rigidity to the fruit tissue against fungal penetration and also can prevent incidence and spread of this disease during storage and transportation. Therefore, Ca dips are recommended for postharvest treatment. Incorporation of calcium gluconate into the chitosan coating formulation was shown to extend the shelf life of strawberries up to 4 days at 20°C with increasing in Ca content of the fruit [87].

CHITOSAN IN COMBINATION WITH NANOCOMPOSITES

Some nanosized substances have been developed to show quanta size, small size, surface, and macroscopic quanta size effects [88]. The complex of zinc (II) and cerium (IV) with chitosan had been applied for preserving Chinese jujube (*Ziziphus jujube*) fruits to expand their shelf life and decrease residues of organophosphorus pesticides on the fruits [89]. The effect of 1% chitosan film with 0.04% nano-silicon dioxide on the qualitative properties of harvested jujube under ambient temperature was investigated. After 32 days, the red index, decay incidence, weight loss, and respiration rate of the coated jujubes were lower compared with those of the control. The lower PAL activity and higher activities of SOD, POD, and CAT of the coated jujubes can be attributed to the compound coating. Increased MDA in the coated jujubes was restrained. Composite coating was shown to be superior in preserving total flavonoid than chitosan coating alone. But no differences were observed in terms of vitamin C loss and total polyphenol content between composite coating and control.

CHITOSAN IN COMBINATION WITH ORGANIC ACIDS

Ochoa-Velasco and Guerrero-Beltrán [90] tested peeled ready-to-eat white prickly pears (*Opuntia albicarpa*) and red prickly pears (*Opuntia ficus-indica*) by submerging them in chitosan solutions containing different concentrations of acetic acid (1.0 or 2.5%) and then storing them for 16 days at 3–5°C and 80–90% RH. For white prickly pear, chitosan coating containing 1% acetic acid delayed weight loss and maintained firmness and color during storage. Most of the sensory values for those coated with chitosan containing 1.0 and 2.5% of acetic acid were higher than those of noncoated fruit. Chitosan coating with 1.0 and 2.5% acetic acid did not affect phenolics content and antioxidant activity. Microorganism population was unchanged in white prickly pears

(<10 CFU/g). Red prickly pears coated with chitosan with 2.5% acetic acid did not maintain their sensory quality during storage and increased their phenolics content and antioxidant activity. Microorganism population increased in red prickly pears (10–500 CFU/g) coated with chitosan during storage.

Coating fresh-cut lotus roots (*Nelumbo nucifera*) with 1% chitosan +1% phytic acid decreased the weight loss and MDA content, delayed browning and reduced the activities of POD, PPO, and PAL, and maintained the content of ascorbic acid and polyphenol; however, the coating caused a bitter astringent taste [91].

Integrated treatment with chitosan (1%) and ascorbic acid (40 mM) helped to retard the rate of respiration and improved the overall quality in plums (*Prunus salicina* cv. “Sanhuali”) after storage at $5 \pm 1^\circ\text{C}$ and 95% RH for 20 days [92]. The color changes were minimized by the inhibition of anthocyanin synthesis by decreasing PAL activity in the fruits treated with chitosan and ascorbic acid. Fruit firmness was maintained by reducing the PG and PME activities on peach fruit during storage [93]. Furthermore, fruit treated with integrated chitosan and ascorbic acid showed higher SOD, POD, CAT, PPO, and POD. Antioxidant enzyme activities reduced the production of superoxide free radicals (O_2^-) and malondialdehyde to delay the senescence in fruits treated with the combined chitosan and ascorbic acid [91].

CHITOSAN IN COMBINATION WITH ANTAGONISTIC MICROORGANISMS

Efficacy tests, using *Bacillus subtilis* ABS-S14 endospores, its crude extract, and chitosan, showed a significant reduction of mandarin fruit postharvest decay caused by *P. digitatum* compared to those found in the presence of individual cyclic lipopeptide antibiotics. It was clearly demonstrated that the *B. subtilis* ABS-S14, itself, its crude extract and chitosan, each induced the activities of POD and PAL in the infected flavedo tissues of mandarin fruit [92].

In the European Union (E.U.), brown rot decay is reported to be controlled by applying good agricultural practices. Exposing fruits to 50°C for 2 h and 95–99% RH helped to reduce the brown rot infection established in the field; however, this treatment failed to protect the fruits against *M. fructicola* infections, becoming more susceptible after the treatment. The application of chitosan (1%) resulted in a preventative control against *M. fructicola*

decay on fruits subjected to preheat treatment. This treatment helped to reduce this disease by 10%, in comparison with the untreated control, which was 73%. However, chitosan applied to wounded fruit had a poor preventive effect and additional preventive control was shown with the application of *B. subtilis* CPA-8 [94].

The effect of chitosan and biocontrol agent has also been shown to be enhanced by the inclusion of food preservatives. Citrus exporting countries are using synthetic fungicides such as Imazalil to control green mold and blue molds during the supply chain. *B. subtilis* ABS-S14, and chitosan induced the activities of POD and PAL in the infected flavedo tissues of mandarin fruit [92]. The combination application enhanced the protection of fruit from *P. digitatum*; however, the use of antibiotic-producing strains of microorganisms in food preservation is limited due to the Food and Drug Administration's (FDA) regulations, because *B. subtilis* ABS-S14 may require specific regulatory approval and registration.

P. expansum causes significant postharvest losses in pear fruit during storage and transportation [95]. The current trend is to develop alternative treatments to replace the synthetic fungicides for postharvest application and biocontrol agents that have been investigated [96,97]. Chitosan coating improved the effect of the biocontrol agent *C. laurentii* on reducing. The combined application of chitosan with *C. laurentii*, and CaCl_2 resulted in 89% inhibition of blue mold decay in pears after 6 days incubation at 20°C, while CaCl_2 (1.0%, w/v) alone failed to show antifungal activity [98].

CHITOSAN IN COMBINATION WITH ETHYL ALCOHOL

Gray mold rot in table grapes is regarded as an economically important postharvest disease, responsible for severe postharvest losses during long-term storage and transportation. At present this disease is controlled with SO_2 fumigation or using sodium metabisulfite impregnated pads within the packaging [99]. Antimicrobial activity of ethanol is well known, and it is a common food additive [93]. Combination treatments with 0.5% chitosan and 10% ethanol reduced the incidence of gray mold rot in table grapes cv. Autumn Seedless and cv. Thompson Seedless during 60 days storage at $0.5 \pm 1^\circ\text{C}$ [23]. The modes of action of chitosan coating was reported based on the protection of the fruit due to its film-forming property [18], and ethanol could have contributed in the synergistic effect due to its antifungal activity.

CHITOSAN AND MINIMALLY PROCESSED FRUIT AND VEGETABLES

Minimal processing, also referred to as fresh-cut, refers to fruit and vegetables that have been cleaned, peeled, cut up, and prepared in such a way that they are ready to eat. The demand for them has been steadily increasing. MAP in combination with chitosan has been used for minimally processed fruit and vegetables. Pushkala et al. [100] investigated a powder coating technique using purified chitosan and chitosan lactate for shelf-life extension of shredded radish (*Raphanus sativus*). Macro perforated low density polyethylene film resealable pouches were used to contain the samples, which were stored at 10°C for 10 days. Chitosan treated samples had a lower weight loss, lower respiration rate, lower titrable acidity, reduced total soluble solids, and higher content of phytochemicals and moisture content compared with control samples. The treated samples also had better sensory acceptability, lower exudate volume, lesser browning, and lower microbial load compared than the nontreated controls. Xiao et al. [101] immersed fresh-cut d'Anjou pear wedges in sodium chlorite solution, followed by coating them with chitosan or carboxymethyl chitosan solutions and storing them at 4°C in unsealed bags. Sodium chlorite inhibited browning and PPO activity and was strongly effective in inactivating *E. coli* O157:H7 on the pear wedges. Coating sodium chlorite treated pear slices with chitosan, however, accelerated the discoloration of cut surfaces and increased the PPO activity. In contrast, coating sodium chlorite treated samples with carboxymethyl chitosan reduced the browning reaction, inhibited PPO activity, maintained tissue firmness, and did not affect weight loss. This led the authors to conclude that carboxymethyl chitosan was more effective than chitosan in this case. Sangsuwan et al. [102] stored fresh-cut cantaloupe (*Cucumis melo*) and pineapple (*Ananas comosus*) slices at 10°C in one of three types of films: a commercial stretch film, an experimental chitosan methylcellulose film and a chitosan/methylcellulose film incorporating vanillin (vanillin film) as a natural antimicrobial agent together with nonwrapped controls. Chitosan/methylcellulose film and vanillin film proved inhibitory to *E. coli* on fresh-cut cantaloupe and the chitosan/methylcellulose film rapidly reduced the number of *Saccharomyces cerevisiae* yeast inoculated onto the cantaloupe and pineapple slices. Pineapple in vanillin film reduced ascorbic acid content and at the end of storage it was only 10% of its original concentration. On mixed lettuce (*Lactuca sativa*), chitosan coating resulted in the development of a bitter taste [19]. The microbiological load on the chitosan dipped lettuce and strawberries were lower, but the antimicrobial effect on lettuce

disappeared after 4 days of storage while it continued on the strawberries during the 12-day storage period.

CONCLUDING REMARKS

The integrated application of chitosan coating with other safe nonpolluting treatments such as physical treatments, antimicrobial agents (botanicals), organic salts and acids, and biocontrol agents, among others is a novel approach to preserve the overall quality of fresh produce by reducing incidence of postharvest diseases, senescence and to extend the storage life numerous horticultural products. The antifungal effect of the integrated treatment during application depends on the inoculum pressure on the fruit at preharvest stage and during postharvest operations. Moreover, the effectiveness of integrated treatments depends on the curative and preventative effect of the treatment. However, prior to commercialization the developed integrated application with chitosan coating needs to be tested for different fresh commodities and between different cultivars within a particular fruit species. Although the previously mentioned integration of two or more treatments can reduce the cost and the amount of chitosan used to develop the edible coating, further research needs to be focused on commercialization, cost benefit analysis, and consumer surveys.

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CHAPTER 6

Use of Chitosan to Control Postharvest Decay of Temperate Fruit: Effectiveness and Mechanisms of Action

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INTRODUCTION

Chitosan and chitin are naturally occurring polymers, present in some plant-pathogenic fungi, especially zygomycetes, and in arthropod exoskeletons [1]. Chitosan is the linear polysaccharide of glucosamine and *N*-acetylglucosamine units joined by β -1,4-glycosidic links, and it is obtained by deacetylation of chitin through exposure to NaOH solutions or to the enzyme chitinase [2]. The degree of deacetylation and the molecular weight of chitosan characterize its properties, such as the number of positive charges of amino groups and therefore, its electrostatic interactions with different substrate at different pH.

Chitosan applications are widespread in many industries, such as cosmetology, food, biotechnology, pharmacology, medicine, and agriculture [3]. For its biocompatibility and biosafety, chitosan has increasing potential as plant protection in the form of a natural fungicide and plant defense booster. It meets the interest of many researchers who use it to prolong the storage of an array of fruit and vegetables worldwide [4].

Chitosan shows a dual mode of action: it shows antimicrobial activity on decay-causing fungi and food-borne pathogens, and induces resistance responses in the host tissues [5]. Moreover, chitosan filmogenic properties make it the ideal compound as an edible coating for fruit, which acts as physical barrier against pathogen infections and is capable of slowing down fruit respiration rate [6].

EFFECTS ON POSTHARVEST DECAY OF FRUIT

After harvest, vegetables and fruits are perishable and susceptible to microbiological spoilage [7]. For these reasons, treatments with fungicides are commonly carried out either in the field or at postharvest. The adverse

effects of synthetic fungicide residues on human health and the environment, and the possibility of the development of fungicide-resistant pathogens, have led to intensified worldwide research efforts to develop alternative control strategies. Among alternatives being investigated to replace the use of synthetic fungicides to control postharvest diseases in fresh produce and to extend their shelf life, chitosan application has been shown to be effective with both pre- and postharvest applications.

Chitosan treatment in the fresh produce industry is safe for the consumer and the environment, and chitosan has been approved by the US FDA as a “generally recognized as safe” (GRAS) food additive. Regulation EU 2014/563 included chitosan chloride as the first member of a basic substance list of plant protection products (planned with Regulation EU 2009/1107), so once commercial compounds are registered, they will officially be included in the list of compounds for plant disease management.

Nowadays, commercial chitosan formulations available on the market are effective for the control of postharvest diseases in different fresh produce commodities. The main differences between practical grade chitosan solutions and commercial chitosan formulations arise from the techniques used for their preparation and application, which is more immediate for the commercial formulations. Indeed, while practical grade chitosan needs to be dissolved in an acid medium some hours before use, the commercial formulations can be quickly dissolved in water [8].

The aim of this chapter is to summarize some of the most recent advances in the application of chitosan in terms of postharvest disease control and maintenance of overall product quality of temperate and citrus fruit.

Table Grapes

Many authors report about the effectiveness of chitosan in reducing postharvest decay of table grapes (*Vitis vinifera*) (Table 6.1), either through preharvest sprays or postharvest applications. In general, for table grapes, preharvest applications are the desired method of treating the bunches, because canopy sprays are easier to integrate into current conventional practices and most table grapes (about 90% in Italy and California) are packed directly in the field [9]. This practice is common due to the fragility of the produce and to other logistic aspects including the higher cost of indoor or shed packing. When table grape bunches were sprayed in the field with solutions of practical grade chitosan at three different concentrations (1%, 0.5%, 0.1%), once (21 days) or twice (21 and 5 days) before harvest, the clusters showed significantly reduced gray mold infections

Table 6.1 Application of chitosan alone or integrated with other treatments to control postharvest decay of table grapes

Variety	Integration of treatments	Stage of application	References
Thompson	—	Preharvest	[11]
Seedless	Ethanol	Postharvest	[18]
Italia	—	Pre- and postharvest	[10]
	—	Postharvest	[22]
Jingxiu	—	Pre- and postharvest	[13]
	Preharvest treatment with <i>C. laurentii</i>	Postharvest	[14]
	Mixed with <i>C. laurentii</i>	Preharvest	[14]
Shahroudi	Putrescine	Postharvest	[20,21]
Muscatel	Bergamot oil	Postharvest	[17]
Muscat	Glucose	Postharvest	[19]
Hamburg			
Red Globe	Grape seed extract	Postharvest	[16]
Autumn	UV-C	Preharvest	[12]
Black			

caused by *Botrytis cinerea* after 30 days storage at 0°C, followed by 4 days of market shelf life [10]. In another study, three different commercial formulations containing chitosan were compared in a field trial in which they were applied four times during the development of Thompson Seedless grapes (berry set, prebunch closure, veraison, and 2 weeks before harvest). The natural incidence of postharvest gray mold after storage at 2°C for 5 weeks was reduced by the chitosan formulations [11]. Berries sprayed with chitosan preharvest showed decreased incidence and severity of gray mold in artificially inoculated fruits, with the best control of gray mold obtained 1–2 days after the application [12]. Postharvest disease has also been reduced by preharvest chitosan treatment and by postharvest UV-C irradiation (0.36 J/cm² for 5 min), with the combination of these treatments providing a synergistic interaction [12]. The combination of chitosan preharvest spray and chitosan postharvest coating results beneficial effects on table grape quality and resistance to decay, superior to the singular application of chitosan [13]. The combination of chitosan with the biocontrol agent *Cryptococcus laurentii* was effective in reducing postharvest table grape decay both when they were used in mixture before the harvest [14] or when postharvest chitosan coating followed preharvest spray of *C. laurentii* [15]. In some trials, chitosan was combined with antimicrobial natural compounds such as grape seed extract [16], bergamot oil [17], or

ethanol [18]. These combinations, while reducing the doses of chitosan, improved also the control of gray mold of table grapes compared to their application alone, and the effect was at least additive and at times synergistic. The complex chitosan–glucose showed better effects on delaying the declines of total soluble solids, ascorbic acid, and titratable acidity; decreasing decay and weight loss; and suppressing respiration rate, in comparison with pure chitosan or glucose [19]. In a similar way, the combination of putrescine and chitosan reduced table grapes weight loss, decay incidence, browning, berry shattering, and cracking [20,21].

Berries

Fruit berries including strawberries (*Fragaria x ananassa*), raspberry (*Rubus idaeus*), blueberry (*Vaccinium* spp.), and currants (*Ribes* spp.) are among the most popular fruits worldwide, rich in nutrients. They are also highly perishable and susceptible, especially during and after harvest, to mechanical injury, fungal spoilage, and desiccation. As with table grapes, strawberry and the other berries are often packed at the field level in order to avoid unnecessary manipulation and eventual mechanical injuries, considering that fruit wounds harbor pathogens and start fruit infections. For this reason, postharvest treatments are avoided while preventive preharvest treatments against storage decay are preferred.

The literature contains numerous successful experimental trials of chitosan applications prolonging fruit berries storage (Table 6.2), and chitosan use in temperate fruit has been investigated since the 1990s. El Ghaouth et al. [23,24], Romanazzi et al. [25], and Zhang and Quantick [26] reported that the control of gray mold and *Rhizopus* rot in chitosan-coated strawberries was similar to synthetic fungicide application. *Cladosporium* spp. and *Rhizopus* spp. infections decreased in artificially inoculated strawberry fruit coated with chitosan and stored at 4–6°C for 20 days [27]. Chitosan coatings of strawberry fruit did not produce taste abnormalities as recorded by trained panelists [28]. When chitosan commercial formulations, easily dissolvable in water, were used as strawberry coatings, their effectiveness in controlling postharvest decay was the same as that of practical grade chitosan [8].

Chitosan coating on strawberry fruit has been used as a carrier to incorporate plant essential oils or extracts that have antifungal activities and nutraceutical properties. Chitosan incorporated with limonene, a major component of lemon essential oils, which has also been given the GRAS status by the FDA, promoted the preservation of strawberry fruit during

Table 6.2 Application of chitosan alone or integrated with other treatments to control postharvest decay of strawberry, raspberry, and blueberry

Fruit	Integration of the treatment	Stage of application	References
Strawberry	—	Postharvest	[8,23–29]
	—	Preharvest	[25,29–31]
	Lemon essential oil	Postharvest	[32]
	<i>Z. multiflora</i> essential oil	Postharvest	[33]
	Carvacrol, cinnamaldehyde, and <i>trans</i> -cinnamaldehyde	Postharvest	[34]
	Red thyme, oregano, limonene, and peppermint essential oils	Postharvest	[35]
	Oleic acid	Postharvest	[36]
	Calcium lactate + calcium gluconate, vitamin E	Postharvest	[37]
	Calcium gluconate	Postharvest	[38,39]
Raspberry	—	Postharvest	[26,40]
	Calcium lactate + calcium gluconate, vitamin E	Postharvest	[37]
Blueberry	—	Preharvest	[40]
	—	Postharvest	[41]

their shelf life [35]. The addition of essential oils of lemon (*Citrus x limon*) [32] or of the plant “Avishan-e Shirazi” (*Zataria multiflora*) [33] enhanced chitosan antifungal activities both in *in vitro* tests and during cold storage of strawberries inoculated with a spore suspension of *B. cinerea*. Coatings based on chitosan either without or with oleic acid at different percentages delayed the appearance of natural fungal infections in comparison to uncoated strawberries [36]. When oleic acid was added to the chitosan coating, there were fewer signs of fungal infection during strawberry storage, especially when the coatings contained the higher levels of oleic acid, which enhanced the antimicrobial properties of chitosan [36]. In another study, chitosan coating embodying essential oils of carvacrol, cinnamaldehyde, and *trans*-cinnamaldehyde were applied to fresh blueberries in order to preserve fresh fruit quality and safety during postharvest storage. These chitosan coatings maintained blueberry firmness and reduced microbial growth on the fresh fruit during storage [34]. When chitosan coating was implemented with calcium on strawberry fruit [37–39], the addition of the salts to the coating formulation enhanced the nutritional value of fruit and was beneficial for the fruit itself. Indeed calcium is considered as a firming agent that improves the texture of fresh fruit, and it has also been reported to delay

the fungal decay of several commodities, including strawberries [8]. Recent experimental trials used edible coatings based on and enriched with chitosan to successfully prolong the shelf life of strawberries. These coatings were composed of chitosan and carboxymethyl cellulose, hydroxypropylmethyl cellulose [42], and beeswax [43].

The literature includes some reports of experimental trials on chitosan application at preharvest level. Strawberries sprayed with chitosan at full bloom or at the green-fruit or whitening fruit stages have shown decreased incidence of gray mold and *Rhizopus* rot infections using natural inocula of *B. cinerea* and *Rhizopus stolonifer*, as seen after 10 days of storage at 0°C followed by 4 days under market simulation conditions. The disease control with 1% chitosan was more effective than chemical fungicides: procymidone and pyrimethanil [25]. Similarly, preharvest treatments with 1 and 2% chitosan decreased the incidence of postharvest gray mold from a natural inoculum, and after preharvest and postharvest inoculation, these applications performed significantly better than a fungicide. This treatment with 1% chitosan also performed better than that with 2% chitosan, which was occasionally phytotoxic [30]. Preharvest spraying with 0.2, 0.4, and 0.6% chitosan decreased postharvest gray mold and maintained the quality of strawberries during storage at 3 and 13°C. Here, the incidence of disease decreased with increased chitosan concentration [31]. In some studies, chitosan effectiveness was cultivar-dependent; indeed chitosan application to strawberries in the field in combination with postharvest dipping of fruits was an effective strategy for controlling gray mold in fruits in cv. Oso Grande but was not in cv. Camarosa [29]. In other trials on raspberries, preharvest treatments were done with hand-spray application per week for 3 weeks, starting when the fruit were just turning pink; alternatively, postharvest treatments were applied immediately after harvest, dipping the fruit in the solutions. In both experiments, the use of chitosan at 1 or 2% was effective in maintaining raspberry quality and retarding respiration, ethylene production, weight loss, and decay incidence [40].

Stone Fruits

The experimental trials with chitosan on stone fruit were mainly carried out on peach, nectarine (*Prunus persica*), and sweet cherry (*Prunus avium*) (Table 6.3). Postharvest trials on sweet cherry showed how a chitosan coating could extend the fruit shelf life, while enhancing the nutraceutical value of sweet cherry. The chitosan film coating retarded the loss of water and the changes in the total polyphenol, anthocyanin, ascorbic acid, flavonoid

Table 6.3 Application of chitosan alone or integrated with other treatments to control postharvest decay of stone fruit

Fruit	Variety	Integration of treatments	Stage of application	References
Peach/ nectarine	Batsch	—	Postharvest	[47]
	—	—	Postharvest	[48]
	—	—	Postharvest	[49]
	Rome star, Andros, Baby Gold 9, Elegant Lady, Venus	Heat treatment	Postharvest	[50]
Sweet cherry	Sweet Heart, Burlat, Blaze Star	—	Pre- and postharvest	[46]
	Ferrovio, Lapins, Della Recca	—	Postharvest	[45]
	—	—	Postharvest	[44]
	Ferrovio	Hypobaric treatment	Pre- and postharvest	[51]

content, and antioxidant capacity of sweet cherry fruits [44,45]. Similarly at preharvest, when 1% chitosan was applied 3 days before harvest, it reduced the incidence of postharvest disease in sweet cherries to the same level as the commercially applied synthetic fungicide fenhexamid [46].

On peach fruit, both chitosan and oligochitosan showed significant effect in controlling postharvest brown rot caused by *Monilinia* spp., preserving valuable quality attributes, and delaying fruit softening and senescence. The effects of chitosan on disease control and quality maintenance of peach fruit was associated with its antifungal and antioxidant property and to the elicitation of defense responses triggered in fruit by chitosan [47–49].

In many experimental trials, chitosan applications on stone fruit were integrated with physical treatments, such as fruit storage at hypobaric pressure or short fruit exposition to warm aqueous vapors. Sweet cherries dipped in 1% chitosan and exposed soon after to hypobaric treatment (0.50 atm for 4 h) showed significant reductions in postharvest natural brown rot, gray mold, and total rot diseases, in comparison with the control and with each treatment applied alone. This combination produced a synergistic effect in its reduction of brown rot and total rots [51]. Similarly, chitosan application 7 days before harvest and postharvest hypobaric treatments at 0.25 or 0.50 atm for 4 h showed synergistic effects in the control of total rot diseases in

sweet cherries stored at 0°C for 14 days, and thereafter held at 20°C for a 7-day shelf life [51]. In another investigation, heating of peaches and nectarines to 50°C for 2 h under 85% relative humidity, followed by the application of 1% chitosan at 20°C, controlled the development of postharvest brown rot and protected the fruit during handling in the packinghouses and until consumer use [50].

Pome Fruits

Experimental treatment of pome fruit with chitosan was carried out mainly in combination with biocontrol agents (Table 6.4).

C. laurentii associated with 0.5% chitosan and calcium chloride was effective for the reduction of postharvest blue mold caused by *Penicillium expansum* in pear (*Pyrus communis*). This combination resulted in more effective mold control than chitosan or *C. laurentii* alone, although chitosan at 0.5% inhibited the growth of the biocontrol yeast *in vitro* and *in vivo*. Moreover, after 6 days of incubation, the combined treatment with *C. laurentii*, chitosan, and calcium chloride inhibited mold decay by nearly 89%, which was significantly higher than the treatments with *C. laurentii*, chitosan, or calcium chloride alone, and with the combinations of *C. laurentii* and chitosan, and *C. laurentii* and calcium chloride [57]. The combination of chitosan and *C. laurentii* on apple (*Malus domestica*) resulted in synergistic inhibition

Table 6.4 Application of chitosan alone or integrated with other treatments to control postharvest decay of pome fruits

Fruit	Variety	Integration of the treatment	Stage of application	References
Apple	Red Delicious	<i>C. saitoana</i>	Postharvest	[52]
		UV-C, <i>C. saitoana</i> , harpin	Postharvest	[53]
	Gala Fuji	Heat treatment	Postharvest	[54]
		Silicon		[55]
Pear	<i>Pyrus pyrifolia</i> Nakai, cv. Shuijing	<i>C. laurentii</i>	Postharvest	[56]
		Calcium chloride, <i>C. laurentii</i>	Postharvest	[57]
	<i>Pyrus bretschneideri</i> Rehd. cv. Yali	Ascorbic acid		[58]
	<i>P. pyrifolia</i> L. cv. Xuehwa	—		[59]

of blue mold rot, which was the most effective treatment at the optimal concentration of 0.1% chitosan [56]. Similarly, the combination of *Candida saitoana* with 0.2% glycolchitosan was more effective in controlling green mold of oranges and lemons than the yeast or glycolchitosan alone [52]. On the contrary, the combination of chitosan with *C. saitoana* or with UV-C had no synergistic effects on the progress of blue mold of apple, although a single treatment provided significant reductions [53].

When chitosan was combined with ascorbic acid as a coating for pear fruit, it delayed the increase of weight loss and retained greater firmness than uncoated fruit; the coatings decreased respiration rate and membrane permeability, and also effectively inhibited core browning after 60 days of storage. Compared with chitosan coating alone, chitosan + ascorbic acid coating increased the beneficial effects and also helped to retain a much higher ascorbic acid content and antioxidative enzymes activity [58]. The combination of chitosan and silicon was effective in reducing brown rot of apple fruit caused by *Monilinia fructicola*, and the effect was greater than that of chitosan or silicon used alone [55].

In another experimental trial, Shao et al. [54] studied the effects of heat treatment at 38°C for 4 days before and after coating apples with 1% chitosan. As well as complete control of blue mold and gray mold on these artificially inoculated apples during storage, chitosan coating followed by heat treatment improved the quality of the stored fruit. Moreover, the presence of chitosan coating prevented the occurrence of heat damage on the fruit surface [54].

When chitosan was used alone, treatments on pear fruit reduced the disease incidence caused by *Alternaria kikuchiana* and *Physalospora piricola* and inhibited the lesion expansion of the two fungi; the disease control effects of chitosan was concentration dependent [59].

Citrus

For citrus fruit (*Citrus* spp.), treatments against postharvest decay are commonly carried out during storage, and postharvest treatments with fungicides are permitted in many countries. The literature contains several examples of citrus coating based on chitosan alone or combined with other substances such as essential oil, microorganisms, or even fungicides (Table 6.5).

Chitosan coating improved firmness, titratable acidity, ascorbic acidity, and the water content for Murcott tangor stored at 15°C for 56 days [69].

Table 6.5 Application of chitosan alone or integrated with other treatments to control postharvest decay of citrus fruits

Produce	Variety	Integration of the treatment	Moment of application	References
Orange	Valencia	–	Postharvest	[60]
	Pera Rio	Fungicides	Postharvest	[61]
	Navel Powell	Bergamot, thyme, tea tree essential oils	Postharvest	[62]
	Washington Navel	<i>C. saitoana</i>	Postharvest	[52]
	Miyagawawase	–	Postharvest	[63]
Tankan citrus fruit	Satsuma	Clove oil	Postharvest	[64]
		<i>R. paludigenum</i>	Preharvest	[65]
		–	Postharvest	[66]
Clementine mandarin		–	Pre- and postharvest	[67]
Shogun mandarin oranges		–	Postharvest	[68]
Murcott tangor fruit		–	Postharvest	[69]
Lemons	Eureka	<i>C. saitoana</i>	Postharvest	[52]

A concentration of 0.2% low molecular weight chitosan was more effective in controlling the citrus decay caused by the fungi *Penicillium digitatum* and *Penicillium italicum* than was the fungicide thiabendazole, exhibiting effective antifungal activity [69]. Application of chitosan on oranges, Valencia variety, reduced the incidence of black spot in the fruit, and it was proposed that the increases in activity of the enzymes chitinase and β -1,3-glucanase 24 h after the chitosan treatment might have contributed to the reduction of the postharvest orange decay [60]. Chitosan treatment significantly reduced the decay of Tankan fruit during storage at 24°C; and after 42 days of storage at 13°C, treated fruits were firmer, exhibited less decay and weight loss than the control fruit [66]. When Clemenules mandarin fruit were treated in the field 86 days before harvest and at postharvest stage with low concentrations of chitosan (0.0125–0.125%) the fruit showed reduced water spot incidence associated with fruit senescence [67].

In many experimental trials with citrus, chitosan was combined with bio-control agents. The combination of the yeast *C. saitoana* with 0.2% chitosan

was more effective in controlling green mold caused by *P. digitatum* of oranges and lemons than the yeast or chitosan alone, and the level of control was similar to that obtained with the fungicide Imazalil on both oranges and lemons [52]. Similarly, preharvest application of chitosan together with *Rhodosporidium paludigenum* showed a more effective control of green mold than the individual treatments and the expression levels of the defense-related genes chitinase and phenylalanine ammonia lyase induced by the treatments increased with decreased disease symptoms [65].

Chitosan coating without or with essential oils (bergamot, thyme, and tea tree oil) was applied to oranges as preventive or curative treatments against blue mold. The addition of the essential oils improved the antimicrobial activities of chitosan, indeed the preventive and curative antimicrobial treatments with coatings containing tea tree oil and thyme were the most effective in reducing microbial growth, as compared to the uncoated samples [62]. Similarly, coatings on navel orange with chitosan and green tea was the best in reducing fruit decay, chilling injury, titratable acidity, and losses percent of juice, compared to the uncoated fruit or fruit that received only a singular treatment [63]. On the other hand, the combination of chitosan and clove oil did not have a synergistic antifungal activity as it was observed in *in vitro* studies. However, the coating of 1% chitosan alone, not combined with clove oil, effectively contributed to the control of green mold on citrus fruit [64]. Chitosan was used in combination with fungicides as well. Chitosan applied with thiabendazole and Imazalil enhanced the potential for controlling black spot lesions on Pêra Rio oranges, while delayed the change of the orange skin color during postharvest at room conditions and did not influence total soluble solids, titratable acidity, pH, and ascorbic acid content. Chitosan can be applied together with fungicides in packinghouses to control citrus black spot and, while maintaining the quality of citrus fruit, it reduced the amount of fungicides used [61].

For citrus fruit, in the scientific community there is increasing interest in the development of edible natural biodegradable coatings, such as chitosan coating, because they could replace the currently used commercial synthetic waxes for maintaining postharvest quality of fruit. With this purpose, some works tested the efficacy of newly developed polysaccharide-based edible bilayer coatings comprising carboxymethyl cellulose and chitosan in preserving postharvest quality of various citrus fruit, including for oranges, mandarins, and grapefruit. Overall, the studies showed that the carboxymethyl cellulose and chitosan bilayer edible coating sufficiently enhanced fruit gloss, but was not effective in preventing postharvest weight loss, and

in mandarins it impaired the flavor quality [70]. However, in another study for the same research group, layer-by-layer coatings based on a combination of two polysaccharides, carboxymethyl cellulose as an internal layer and chitosan as an external layer, provided mandarins with the high firmness, low weight loss, and satisfying gloss without affecting natural flavor and the respiration process [71].

MECHANISMS OF ACTION

The summarized studies have shown that chitosan can effectively maintain the fruit quality and can control postharvest decay during storage and shelf life. The film-forming properties, antimicrobial activities, and induction of plant resistance of chitosan appear to be the main factors in chitosan success. With its intrinsic properties, and because of its double activity on the host and the pathogen, chitosan can be considered as the first of a new class of plant-protection products [72,73]. However, more studies concerning the chitosan's exact mechanisms of action are needed, including aspects related to its antifungal and antibacterial activities, which remain unclear.

The following sections provide some information on antimicrobial, eliciting, and film-forming properties of chitosan.

Antimicrobial Properties

The antimicrobial activities of chitosan appear to rely on electrostatic interactions between positive chitosan charges and the negatively charged phospholipids in the fungal plasma membrane. The cationic charge of chitosan is due to protonation of the amino group at C2 position, caused by the deacetylation of chitin to chitosan. The degree of deacetylation influences the number of positive charges eventually present on the chitosan molecule: chitosan with high degree of deacetylation has more numerous positive charges.

The antifungal activity of chitosan have been reported against the main postharvest fruit pathogens such as *B. cinerea* [24,52,66,74,75], *R. stolonifer* [24,76], *Penicillium citrinum* [77], *P. digitatum* [66], *P. italicum* [66], *P. expansum* [52,56,75], *M. fructicola* [48,55], *A. kikuchiana* [59], *P. piricola* [59], and *Aspergillus niger* [78].

When chitosan faces a fungal cell, chitosan first binds to the target membrane surface and covers it; and in a second step, after a threshold concentration has been reached, chitosan causes membrane permeabilization and the release of the cell contents [79]. As chitosan is applied, the homeostatic

mechanism of the fungal cell becomes drastically transformed, because as chitosan forms channels in the membrane, it allows the free passage of Ca^{2+} down its gradients, which cause instabilities in the cells that can lead to the death of the cell itself [80]. When *R. stolonifer* was grown in media containing chitosan, the release of proteins by the fungal cells increased significantly. It was proposed that this release of proteins from the fungal cells was because there were sites where the cell membrane was damaged by chitosan [81]. Besides its ability for membrane permeabilization, chitosan can also penetrate into fungal cells. Fluorescent labeled chitosan was detected in fungal conidia and it was hypothesized that chitosan itself permeabilizes the plasma membrane to allow its entry into the cytoplasm [80,82]. A study used fluorescence visualization to demonstrate that oligochitosan can penetrate the cell membrane of *Phytophthora capsici* and *A. niger*, and that, as it is positively charged, chitosan can bind to intracellular targets, such as DNA and RNA, which are negatively charged [78,83].

Several studies have described the morphological changes on fungal hyphae and reproductive structures that can be induced by chitosan. Scanning electron microscopy observations of *Fusarium sulphureum* treated with chitosan have revealed effects on hypha morphology. The growth of hyphae treated with chitosan was strongly inhibited, and they were tightly twisted and formed rope-like structures [84]. Examination of ultrasections of the hyphae and conidia of chitosan-treated *Alternaria alternata* revealed marked alterations to the cell wall. The chitosan-treated mycelia showed predominantly loosened cell walls, and in some areas, there was also lysis. The conidia exposed to chitosan were intensely damaged, and usually eroded, with broken cell walls seen that contained in some cases no cytoplasm [85].

Eliciting Properties

Plant resistance toward pathogens occurs through hypersensitive responses that result in cell death at the penetration site, structural alterations, accumulation of reactive oxygen species (ROS), synthesis of secondary metabolites and defense molecules, and activation of pathogenesis-related (PR) proteins [86]. The application of external elicitors to vegetative tissue can trigger plant resistance by simulating the presence of a pathogen. Several studies have reported that chitosan can induce a series of enzyme activities and the production of various compounds that are correlated with plant defense reactions to pathogen attack [4,72].

Chitosan can increase PR gene function through multiple modes, which includes activation of cell surface or membrane receptors, and

internal effects on the plant DNA conformation, which can, in turn, affect gene transcription [87]. Histochemical staining of chitosan polymers indicates that chitosan accumulates in the plant cell wall, cytoplasm, and nucleus. The accumulation of positively charged chitosan along with its high affinity for negatively charged DNA suggests that it has a direct effect on the regulation of plant defense responses, with influences on mRNA and protein synthesis [88].

Phenylalanine ammonia lyase (PAL) is the key enzyme in the phenol synthesis pathway [89], and the accumulation of phenols that act as phytoalexins is considered the primary inducible response in plants against a number of biotic and abiotic stresses [90]. Chitosan application has been reported to increase PAL activity in treated fruit tissue. Table grape bunches with preharvest spraying with chitosan showed a threefold increase in PAL activity in the berry skin 24 h and 48 h after chitosan application [10]. PAL elicitation by chitosan was confirmed with table grapes sprayed in the vineyard with or without *C. laurentii* and coated with chitosan at post-harvest, and then stored at 0°C [13–15]. Chitosan treatments induced the activity of PAL in sweet cherry [44] and strawberry [25,91], thus enhancing the fruit defense responses.

Chitinase and β -1,3-glucanase are two PR proteins that participate in defense against pathogens, because they can partially degrade the fungal cell wall [86]. Increases in the activities of chitinase and β -1,3-glucanase were demonstrated as a result of chitosan application in Valencia oranges [60]. Similarly, in raspberry and strawberry, chitosan induced a significant increase in chitinase and β -1,3-glucanase activities, as compared to the controls [26,91,92]. In table grapes, preharvest chitosan treatments from three different commercial formulations induced the activity of endochitinase, while two of the chitosan formulations induced exochitinase activity [11].

Chitosan treatment might induce fruit disease resistance through regulation of ROS levels, antioxidant enzymes, and the ascorbate–glutathione cycle. Changes in the content of ROS, such as H_2O_2 and O_2^- , are the earliest events that correlate plant resistance to pathogens [93] since ROS are involved in the development of disease resistance in fruit [94]. Chitosan application was reported to reduce ROS and the hydrogen peroxide content in tissues of treated fruit, such as pear [95], table grapes [11], and strawberry [8]. This might be due to direct effects, as chitosan itself has antioxidant activity and scavenges hydroxyl radicals [96], or to indirect effects, such as chitosan inducing the plant antioxidant system.

The presence of antioxidants, such as the phenols, can substantially reduce the ROS content of plant tissues, and their hydroxyl groups and unsaturated double bonds make them susceptible to oxidation [97]. Moreover, phenolic compounds are involved in plant responses against biotic and abiotic stresses [90,98]. Chitosan coating was effective in the intensification of total antioxidant capacity of treated apricot (*Prunus armeniaca*), with increases in the phenolic compounds in the fruit tissue [99]. Table grapes treated with chitosan had higher phenolic compound contents [11,21].

Chitosan treatment has been reported to influence antioxidant enzyme activities in the tissues of fruits and vegetables. Compared to untreated strawberries, those treated with chitosan maintained higher levels of antioxidant enzyme activities, such as catalase, glutathione-peroxidase, guaiacol peroxidase, dehydroascorbate reductase, and monodehydroascorbate reductase [92]. Compared to the tissue of uncoated fruit, higher activities of superoxide dismutase, catalase, and peroxidase were reported after chitosan application to pears [58,95]. In addition, increased peroxidase activity after chitosan application has been reported for several other commodities, such as table grapes [13], pears [59], sweet cherries [44], and oranges [60].

Film-Forming Properties

When a fruit is dipped in a chitosan solution, a semipermeable film around the fruit surface is produced after drying. The coating modifies the internal atmosphere by reducing oxygen and/or elevating carbon dioxide levels, which decreases the fruit respiration level and metabolic activity, and hence delays the fruit ripening and senescence processes [6,100,101]. A suppressed respiration rate slows down the synthesis and the use of metabolites, which results in lower soluble solids due to the slower hydrolysis of carbohydrates to sugars [102].

The chitosan coating with its filmogenic properties has been used as a water barrier, to minimize water and weight loss of fruit during storage [102,103]. All of these physiological changes have been reported in fruit and vegetables treated with chitosan. Chitosan treatments of pears during storage reduced their vital activities, and in particular, their respiration rates, which result in reduced weight loss, keeping the fruit quality and prolonging the shelf life [104]. Strawberries treated with chitosan alone or in combination with calcium gluconate showed reduced weight loss and respiration, delaying the ripening and the progression of decay [39]. Chitosan

combined with bergamot oil provided a water vapor barrier for cold-stored table grapes, which reduced fruit weight losses [17].

Firmness is a major attribute that dictates the postharvest quality of fruit [105]. Fruit softening is a biochemical process that is normally attributed to the deterioration of the cell-wall composition, which involves the hydrolysis of pectin by enzymes (e.g., polygalacturonase) [106]. Low levels of oxygen and higher levels of carbon dioxide restrict the activities of these enzymes and promote the retention of fruit firmness during storage [107]. Moreover, due to reduced transpiration, the water retention provides turgor to the fruit cells. Chitosan coatings had beneficial effects on strawberry firmness, such that by the end of the storage period, the treated fruit had higher flesh firmness values than the untreated ones [39]. In several studies, chitosan coating maintained the firmness during storage of table grapes [16,17], apples [54], pears [58], and peaches [49].

Chitosan coating delayed color changes in citrus [61] and strawberry [37,39,108]. The delay of color development for the fruit treated with chitosan can be attributable to the slow rate of respiration and reduced ethylene production, which leads to delayed fruit ripening and senescence. Sensory analyses also revealed beneficial effects of chitosan coating in terms of delaying rachis browning and maintaining the visual aspects of table grape berries [16,17].

Chitosan coatings can be used as a vehicle for carrying functional ingredients, such as other antimicrobials, minerals, antioxidants, and vitamins. Some of these combinations can enhance the effects of chitosan or reinforce the nutritional value of the commodities [101]. Chitosan-based coatings were used to incorporate calcium or vitamin E, increasing the content of these nutrients in fresh and frozen strawberry and raspberry. Incorporation of calcium or vitamin E into chitosan-based coatings did not alter its antifungal properties, while it enhanced the nutritional value of these fresh and frozen strawberries and raspberries [37].

In the food industry, chitosan shows potential for application to food packaging, as a surrogate for petrochemical based films and as an innovative environmentally friendly material based on its physicochemical properties, its biodegradability, and its antifungal and antibacterial properties, with non-toxic and nonresidual effects [109,110]. Considering the health-conscious consumers and the carbon footprint on the environment, modern food packaging needs to address the application of bio-based active films or biopolymers, and chitosan shows potential as a bioagent or additive for the preparation of active films.

CONCLUDING REMARKS

This chapter summarizes some studies showing that chitosan can effectively maintain fruit and vegetable quality and can control postharvest decay during storage and shelf life.

Chitosan is considered a biopolymer safe for humans and the environment, and it is known for its antimicrobial activity as well as eliciting plant defenses [111]. Multicomponent edible coatings based on chitosan can provide the desired barrier protection for fruit, while also serving as a vehicle for the incorporation of specific additives such as minerals, vitamins, nutraceutical compounds that can enhance the nutritional value of the commodities and the chitosan functionality as antioxidant or antimicrobial agent against food-borne microorganisms and fruit pathogens [112].

Although a lot of information regarding the effectiveness of chitosan in the control of postharvest decay of fruit and vegetables is available, its application to large-scale tests and its integration into commercial agricultural practices are key points that need to be investigated further. However, several mechanisms relating to its antimicrobial and eliciting activities remain not completely understood. New knowledge about these aspects will provide the necessary information to support decisions relating to the preparation of the chitosan, which molecular weight and degree of deacetylation to use, and the kind of commercial formulations that are most effective.

The availability of commercial chitosan products that are easily dissolvable in water now provides an alternative to synthetic fungicides for growers, for the control of diseases of fruit and vegetables. The recent EU Regulation 2014/563 that included chitosan chloride in the list of plant protection products can further increase the interest and use among growers, stimulating new studies concerning chitosan application, its integration into current agricultural practices, and its modes of action.

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CHAPTER 7

Chitosan and Its Derivatives as Active Ingredients Against Plant Pests and Diseases

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INTRODUCTION

Pests and pathogenic microorganisms that attack plants are of great economic importance around the world. They induce decay and harmful effects on most of the agricultural crops during the growing season and postharvest stage. The success of the current farming practices is mainly due to discovery and application of synthetic pesticides for controlling pests and pathogens. The significant increase in crop yields related with the green revolution would not have been possible without incorporation of synthetic pesticides in integrated pest management (IPM) programs [1,2]. However, continuous use of these products has given rise to two major problems: public concern regarding contamination of the products with high residue of pesticides, and the appearance of resistant strains [2–5]. Moreover, concerns over the potential impact of pesticides on the environment are now more pressing, and registration procedures are becoming more stringent. These new regulations have reduced the number of available pesticides in some agricultural sectors. Therefore, the development of environmentally compatible pest and disease control agents are important because they could partially replace the harmful pesticides withdrawn for economic or regulatory reasons [6–10]. Alternative methods for disease and pest control include both biocontrol agents and biologically derived pesticides. Natural products offer advantages including their ability to target microorganisms and pests, along with their unique modes of action with low mammalian toxicity. Furthermore, they are generally biodegradable and do not persist in the environment. Among the biologically active products, the biopolymer chitosan has recently attracted more attention because of its unique characteristics and biological activities [9–13]. Chitosan is a linear copolymer of glucosamine (GlcN) and *N*-acetylglucosamine (GlcNAc) units linked by 1,4-glucosidic bonds and can be obtained through the alkaline hydrolysis of chitin extracted from

marine crustaceans and cell walls of fungi [13–15]. While being a natural resource, chitin has not been used for a long time, and now it is meeting the interest in chitosan and chitooligosaccharides. The properties of chitosan, including a defined chemical structure, physical and biological activity, and chemical and enzymatic modification, make this polymer advantageous for numerous applications in many fields [6,9,10,16–18]. From a biological activity standpoint, chitosan compounds are attractive for agriculture applications because they are safe to humans and the environment. Due to its high biological activity, biodegradability, biocompatibility, and non-toxicity to mammals and nontarget organisms, chitosan is widely used as a biocide either alone or blended with other natural products [10,16,19–23]. To broaden chitosan's pesticidal applicability against plant diseases and pests, comprehensive knowledge of its activity is necessary.

This chapter covers some recent findings on use of chitosan-based products in pest and disease management. In addition, we consider the new technological applications of chitosan products in agriculture and perspectives on future studies in this field. Modes of action of these products against several target pathogenic microorganisms and pests are also discussed.

CHEMISTRY AND PROPERTIES OF CHITOSAN

Chitosan is a linear biopolymer that consists of GlcN and GlcNAc units linked by β -1,4-glucosidic bonds. It is obtained through deacetylation process of purified chitin using hot alkali [24–27]. The alkali treatment produces 40–80% deacetylated chitosan; thus, chitosan is a heteropolymer having GlcN and GlcNAc units together of the original chitin in polymeric chain. An important parameter to examine the chitosan structure and properties is the degree of N-acetylation (DA), which is the ratio of GlcNAc to GlcN units. In chitin, the DA is ≥ 0.80 , where chitosan is completely or partially N-deacetylated derivative with a DA lower than 0.20. To calculate the DA, many studies have been performed with several analytical techniques, including IR and UV spectroscopy, elemental analysis, gel permeation chromatography, ^1H - and ^{13}C -NMR spectroscopy, thermal analysis, and various titration methods [28–35].

Whereas other biopolysaccharides are acidic in nature, both chitin and chitosan are basic and as such undergo the typical neutralization reactions of alkaline compounds [36,37]. At a molecular level, chitin and chitosan appear similar because have reactive hydroxyl groups. However, chitosan is more accessible to many reagents due to the presence of free amino groups. While there are few solvents for chitin, nearly all aqueous acids dissolve chitosan,

formic and acetic acids being the most commonly used [37–39]. In acidic pH, the amino groups of chitosan can be protonated resulting soluble product in water. The solubility depends on the free amino groups and the DA, and generally aqueous acetic acid solutions (0.5–3%) are used as the best solvents of chitosan.

Chitosan is like other polysaccharides, susceptible to a variety of degradation processes, including oxidative-reductive, free radical depolymerization, and acid and enzymatic hydrolysis. Acid hydrolysis effectively cleaves the glycosidic linkages of chitin and chitosan main chains, lowering their molecular weight (MW) [40]. Resulting low MW chitooligosaccharides are β (1→4) linked homo- or heterooligomers of GlcNAc and/or GlcN with a chain length of up to $n = 15$ and a MW of up to 10 kDa [41–43]. Unlike chitin or chitosan, chitooligosaccharides have a lower viscosity with greater solubility in water at neutral pH due to their shorter chain length and free amino groups in GlcN units [43]. These properties have made them attractive targets for many applications in medical, agriculture, food, and biotechnological fields [9,10,43,44].

Chitosan has three reactive functional groups: two hydroxyl groups on C-3 and C-6 in each repeating unit, and the amino group on C-2 in each deacetylated unit (Figure 7.1). These groups are reactive to chemical modifications to enhance mechanical, physical, and biological properties. The most common reactions occurring on the hydroxyl groups are etherification and esterification [45,46]. Selective O-substitution can be performed by protecting the amino groups during the reaction [47–49]. The presence of nucleophilic amino groups allows selective N-substitution, such as N-alkylation or N-arylation and N-acylation by reacting chitosan with alkyl or aryl halides and acid chlorides, respectively [47,50–53]. Reductive amination reaction is the most reliable procedure for preparation of Schiff bases of chitosan and N-alkyl or -aryl chitosan derivatives, wherein the amino group

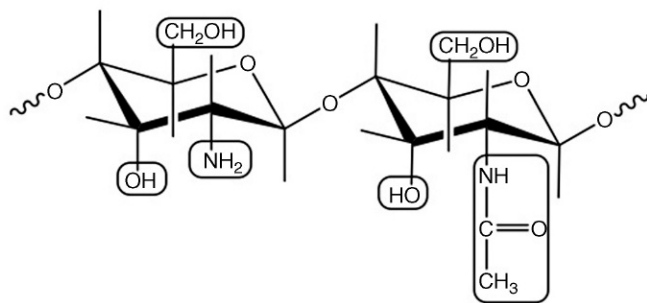


Figure 7.1 Illustration of the Possible Reaction Sites in the Molecule of Chitosan.

is converted to an imine (Schiff base) with an aldehyde or ketone, and subsequently reduced by sodium borohydride or sodium cyanoborohydride to form *N*-alkyl or -aryl derivatives [38,54–58]. These reactions are facile, and the degree of substitution (DS) is generally high.

Because of the insolubility of chitosan in organic solvents and water, direct quaternization of chitosan has been proved [59–64]. Quaternary ammonium chitosan salts, a kind of chitosan derivatives with permanent cationic charges on the polysaccharide backbone, have attracted much attention to be used as antimicrobial agents and drug delivery carriers. They can be obtained by two different ways: by addition of a substituent, which contains a quaternary ammonium group [64], and by quaternization of amino groups of chitosan by means of a suitable alkylating agent [63]. The first possibility uses quaternary ammonium epoxides such as glycidyltrimethylammonium chloride. The second one, quaternization of chitosan itself, utilizes alkylchlorides or dialkylsulfates such as dimethyl- or diethylsulfate.

BIOLOGICAL ACTIVITY OF CHITOSAN AGAINST PLANT DISEASES AND PESTS

Effect on Bacterial Diseases

To date, some studies have reported bactericidal and bacteriostatic effects of chitosan and its derivatives against plant pathogenic bacteria [6,8–10, 12,16,19,65,66]. The minimum inhibitory concentration (MIC) of chitosan varies among species from 10 mg/L to 5000 mg/L, depending on the MW, the pH, chemical modification, DA, DS, and the type of microorganism [6,9,67–70]. Most of the antibacterial effects have been investigated against *Agrobacterium tumefaciens*, *Corynebacterium fascians*, *Erwinia amylovora*, *Erwinia carotovora*, *Pseudomonas solanacearum*, *Pseudomonas syringae*, *Sarcina lutea*, and *Xanthomonas axonopodis*, although most of these studies were conducted *in vitro* [71–74]. Chitosan showed strong *in vitro* antibacterial activity against twelve *Xanthomonas* spp. isolated from poinsettia tree (*Euphorbia pulcherrima*) [74]. The authors indicated that chitosan with degree of deacetylation 85.3 and MW 11.29×10^5 Da reduced bacterial growth up to 61.88% at 100 mg/L after 24 h of incubation. As well, Li and coworkers [73] found that the same type of chitosan at 200 mg/L had strong activity against nine strains of *Xanthomonas* spp. that cause leaf spot disease of *E. pulcherrima*.

Two kinds of chitosan, with different MW and DDA, possess a strong antibacterial activity against bacterial leaf blight and streak in rice (*Xanthomonas oryzae*) and significantly reduced the incidence and severity of

disease compared to control under greenhouse conditions [66]. In this study, the interaction between chitosan and rice pathogens was affected by chitosan type and concentration, the bacterial species and the contact time. Yang and coworkers [75] examined the inhibitory effect and mode of action of chitosan against *Acidovorax avenae* that causes rice bacterial brown stripe disease. This study indicated that chitosan at 100, 200, and 400 mg/L inhibited the *in vitro* growth and the bactericidal efficiency generally increased with increasing concentration and incubation time. The antibacterial activity in this study was mainly due to cell membrane damage, which was evidenced by cell lysis and the increased release of cell materials.

The antibacterial activity of three MWs chitosan products (3.60×10^5 , 6.11×10^5 , and 9.53×10^5 Da) was tested *in vitro* against *A. tumefaciens*, *C. fascians*, *E. amylovora*, *E. carotovora*, *P. solanacearum*, and *S. lutea* [71]. The data indicated that chitosan had a direct effect on bacterial cells and the activity increased with the increase of the MW. In contrast, other three MWs chitosan (0.5×10^4 , 3.7×10^4 , and 5.7×10^4 Da), prepared from a commercial product of 2.9×10^5 Da, showed that the lowest MW (0.5×10^4 Da) exhibited the highest activity against *A. tumefaciens* (MIC = 2600 mg/L), while chitosan 3.7×10^4 Da was the most active against *E. carotovora* (MIC = 950 mg/L) [76]. The activity increased with the decrease in the MW, as also reported by other researchers [6,11,19,43,77] who concluded that the oligochitosans (degree of polymerization, DP 2–30) produced from chitosan hydrolysis were not only water-soluble but also were more effective than native chitosan. Another study demonstrated that the chitosans MW $\leq 10^4$ Da, had greater antibacterial activity than high MW chitosans; however, a DP of at least seven was required [78]. Coqueiro and Di Piero [79] found that three commercial MWs chitosan (low, medium, and high) showed similar effect on the development of leaf spot of tomato caused by *Xanthomonas gardneri* under greenhouse conditions. It was reported that chitosan at 1000 and 3000 mg/L reduced leaf spot severity by 70% when applied up to 3 days before inoculation. Lu et al. [80] found that the mixed solutions of raw bamboo vinegar and chitosan had better antibacterial activity than did raw bamboo vinegar alone against *Ralstonia solanacearum*. High DDA (78%) and concentration (1500 mg/L) of chitosan resulted in better inhibition efficiencies than bamboo vinegar. The field efficacy of chitosan was compared with copper (hydroxide + oxychloride) in controlling bacterial canker of kiwifruit, caused by *P. syringae* [81]. Both compounds were sprayed every 14 days, from early April to early June, and once a month, from mid-November to mid-February. Chitosan did not induce

phytotoxicity in plants and fruits and showed an overall higher level of performance than copper product to reduce symptoms of the disease by testing it during three years.

To enhance the antibacterial activity of chitosan, several research groups have been modifying its structure by grafting biologically active molecules or by increasing its solubility in neutral pH. Grafting the alkyl chains or aryl moieties into chitosan significantly increased its antibacterial activity against plant pathogenic bacteria [54,61,82–84]. However, this activity is limited to acidic conditions due to its poor solubility above pH 6.5, where chitosan starts to lose its cationic nature. Therefore, many studies have focused on the preparation of chitosan derivatives water-soluble over a wide pH ranges and tested against plant pathogenic bacteria [55,59,60,85]. Among water-soluble chitosan derivatives, the quaternary ammonium chitosan, prepared by introducing a quaternary ammonium group into a dissociative hydroxyl group or amino group of the chitosan, exhibited stronger antibacterial activity than chitosan on a range of pH values.

A series of *N*-(benzyl) and quaternary *N*-(benzyl) chitosan derivatives was synthesized and their antibacterial activities were evaluated *in vitro* against *A. tumefaciens* and *E. carotovora* [61]. MICs of *N*-(benzyl) chitosan derivatives were in a range of 800–1780 mg/L against *A. tumefaciens* and 700–1550 mg/L against *E. carotovora*. However, the MICs of quaternary *N*-(benzyl) chitosan derivatives were in a range of 500–875 mg/L against *A. tumefaciens* and from 600 mg/L to 975 mg/L against *E. carotovora*. These data confirmed that grafting of benzyl moiety or quaternization of the derivatives onto chitosan molecule was successful in inhibiting bacterial growth. Moreover, increased water solubility of the compounds by quaternization significantly increased the activity. These compounds also inhibited some of exocellular enzymes, including polygalacturonase and pectin-lyase at 1000 mg/L. Badawy and Rabea [55] added *N*-(4-carboxybutyryl) chitosans at different DS (0.10–0.53), and obtained MICs of 800–1200 mg/L against *A. tumefaciens* and 725–1100 mg/L against *E. carotovora*. The inhibitory effects differed with regard to DS and type of bacteria. Increasing DS led to increased bacterial activity, where *N*-(4-carboxybutyryl) chitosan of 0.53 DS displayed the highest inhibition activity against *A. tumefaciens* and *E. carotovora* with MICs of 800 and 725 mg/L, respectively. Considering the susceptibility of the tested microorganisms, it was noticed that *E. carotovora* was more susceptible to these compounds than *A. tumefaciens*, and it may be attributed to differences in the cell wall constitution [86–88].

Effect on Fungal Diseases

Chitosan is often used to control fungal diseases of plants as an elicitor rather than a direct toxic agent because it has been reported that chitosan can activate plant defenses to the infected plants including grape [89–91], wheat [92–93], barley [94], pearl millet [95], cucumber [96], carrot [97], potato and tomato [98,99], sunflower [100], rice [101,102], and coconut [103]. Fungi of *Cylindrocladium floridanum*, *Cylindrocarpon destructans*, *Fusarium acuminatum*, and *Fusarium oxysporum*, which attack forest nurseries, were also inhibited by chitosan [104]. Similarly, *Aspergillus flavus* was completely inhibited in field-growing corn and peanut [105]. Chitosan causes morphological changes, structural alterations, and molecular disorganization of fungal cells that reflect its fungistatic or fungicidal potential [6,9–11,16,19,106–108]. The MICs of chitosan and its derivatives varied between 10 mg/L and 500 mg/L [6,9,10,107–116]. In general, chitosan applied at 1000 mg/L is capable of reducing the growth of several fungi, except those containing a chitosan molecule in their cell walls such as zygomycetes [117]. The oomycete pathogens such as *Phytophthora capsici* in peppers have also been controlled by chitosan treatment [118] and *Phytophthora infestans* in potato [119].

Fungal inhibition by chitosan has been observed on different stages of growth such as the mycelia, spore formation, spore viability and germination, and production of fungal virulence factors. It has been generally recognized that the antifungal activity depends on the concentration applied, MW, DA, pH of the chitosan solution, viscosity, and the target microorganism [117]. The maximum antifungal activity of chitosan is often observed around its pK_a (pH 6). One isolate of *Cytosporina* sp. was completely inhibited at 75 mg/L of chitosan; however, another isolate of the same genus was unaffected by 1000 mg/L. Chitosan of 9.2×10^4 and 3.57×10^5 Da and 94.2% DDA caused 25–90% mycelial growth inhibition in *Penicillium digitatum*, *Penicillium italicum*, *Botrydiplodia lecanidion*, and *Botrytis cinerea* [120]. Benhamou et al. [110] reported that chitosan at 500 mg/L and 1000 mg/L was completely effective in reducing the disease incidence caused by *F. oxysporum* f. sp. *radicis-lycopersici* in tomato plants. At the same time, El Ghaouth and his research group [121] revealed that the mycelial growth of *Pythium aphanidermatum* was inhibited by 400 mg/L chitosan. El Ghaouth and coworkers [111] reported that chitosan markedly reduced the radial growth of *B. cinerea* and *Rhizopus stolonifer*, with a greater effect than N,O-carboxymethyl chitosan, polygalacturonate or D-glucosamine, and its inhibitory activity appeared to increase with the level of deacetylation.

In addition to inducing cellular leakage of amino acids and proteins, chitosan also caused morphological changes in *R. stolonifer*. The ultrastructural study showed that chitosan caused deep erosion of the cell wall as well as increasing the cell wall thickness. Hernández-Lauzardo et al. [122] reported that the mycelial growth and sporulation of *R. stolonifer* and *Mucor* spp. were inhibited at different concentrations (500, 1000, 1500, and 2000 mg/L) of chitosan, with the best results when 2000 mg/L were applied. They found that the spore morphology varied according to the fungus evaluated; *R. stolonifer* was more susceptible and spores showed variations in area, form, and optical density (2000 and 1500 mg/L), while *Mucor* spp. showed variations only in optical density starting at 1500 mg/L. Our research group found that the mycelial growth of *Alternaria alternata*, *B. cinerea*, *Colletotrichum gloeosporioides*, and *R. stolonifer* was significantly reduced at chitosan concentrations of 750–6000 mg/L [71]. The same observation was found on *Sclerotinia sclerotiorum* when chitosan tested was at concentrations ranging from 10 g/L to 40 g/L [123]. Other studies showed a linear decrease of *Rhizoctonia solani* growth as the concentration gradually increased from 500 mg/L to 6000 mg/L [124]. However, complete inhibition was recorded against *F. oxysporum*, *R. stolonifer*, *P. digitatum*, and *C. gloeosporioides* at 30000 mg/L [125,126]. The effect of the MW on the antifungal activity has been widely explored: however, researchers used uncertain low MW chitosan term for a partially depolymerized chitosan, not indicating exactly its MW [127]. Badawy [76] confirmed that the antifungal activity was increased with the decrease of the MW, where a chitosan of low MW (0.5×10^4 Da) exhibited the highest antifungal potency against *Botrytis fabae*, *F. oxysporum*, and *R. stolonifer* compared to chitosan of 3.7×10^4 Da. This finding was in agreement with other studies [43,77,114,128–131] reporting that oligochitosans, as lower in MW than chitosan, have proved to be more effective than chitosan. Qiu et al. [132] found that low and high MW chitosan efficiently inhibited the radial growth of *F. concentricum* at 4000 mg/L, which appeared to be more effective in inhibiting spore germination and germ tube elongation than that of the carboxymethyl chitosan. In particular, spore germination was totally inhibited by low and high MW chitosan at 50 mg/L. However, no differences were observed in the fungicidal pattern among the three different MW chitosans, whereas no superior effect resulted from increasing the concentration from 5 g/L to 20 g/L [133].

Chitosan depolymerization can be carried out by various techniques include chemical and enzymatic degradation. It has been reported that the depolymerized products have stronger antifungal activity against plant

pathogens than the original chitosan. For example, Falcón and coworkers [134] studied the enzymatic degradation of chitosan with Pectinex Ultra SPL (Novozymes A/S, Bagsvaerd, Denmark) to obtain protective agents against *Phytophthora parasitica nicotianae* in tobacco plants. They found that the 24 h hydrolysate showed the highest antifungal activity, while the native chitosan was the lowest. In this study, the *in vitro* growth inhibition of several *P. parasitica* strains by two chitosans of different DAs was compared. While low acetylated chitosan (DA 1%) completely inhibited three *P. parasitica* strains at 500 mg/L and 1000 mg/L; however, chitosan with DA 36.5% did not inhibit completely these strains [134]. Chitosans having a 45–1400 DP have clearly demonstrated that the antifungal activity increased with the decrease of the MW. This was consistent with the study of Oliveira [135] who found that the same trend was observed with a series of polyglucosamines having a DP from 20 to 2500. However, when comparing the activities of chitosans having a DP value higher than 2500, it was found that they were more active than their low DP counterparts. Interestingly, oligochitosans are effective in inhibition of spore germination, germ tube elongation, and mycelial growth of fungal phytopathogens, including root and necrotrophic pathogens such as *Alternaria solani* [118], *B. cinerea* [120], *Fusarium* [118], *R. stolonifer* [114], *Penicillium* [136], *P. capsici* [118], and *Sclerotium rolfsii* [137]. Oligochitosans were more effective than chitosan in inhibiting mycelial growth at different stages in life cycle of *A. alternata*, *Alternaria kikuchiana*, *A. solani*, *B. cinerea*, *Colletotrichum orbiculare*, *Exserohilum turcicum*, *Fusarium graminearum*, *F. oxysporum*, *P. capsici*, *Phomopsis fukushi*, *Physalospora piricola*, *Pyricularia oryzae*, and *Verticillium dahlia* [118,138,139].

The effect of DA on the antifungal activity of chitosan was studied and it was found that the activity of chitosan increased with the decrease in the DA from 0.70 to 0.12. The decrease in the DA means an increase of the free amino groups on the chitosan that protonated under acidic conditions and leads to an increase the interaction between chitosan and negatively charged cell walls of microorganisms [140]. A complete deacetylated polyglucosamine exhibited the highest activity against *A. alternata*, *B. cinerea*, *Penicillium expansum*, and *R. stolonifer* with MIC of 100–1000 mg/L [135,141]. This effect was also observed by Pacheco et al. [142] who evaluated five chitosans with different DA against *P. digitatum*.

The chemical modification of chitosan is continuously studied because it has the potential to provide an antifungal activity against plant pathogenic fungi. Several research groups have started the chitosan molecule modification to produce high antifungal active compounds (Table 7.1).

Table 7.1 Selected chitosan derivatives tested against plant pathogenic fungi

Chitosan derivatives	Tested fungi	References
2-(α -Arylamino phosphonate) chitosans	<i>A. solani</i> , <i>C. gloeosporioides</i> , <i>F. oxysporum</i> , and <i>Phylllosticta zingiberi</i>	[151]
Acyl thiourea chitosans	<i>A. solani</i> , <i>A. fumigatus</i> , <i>C. gloeosporioides</i> , <i>F. oxysporum</i> , <i>F. solani</i> , <i>Geotrichum candidum</i> , <i>P. zingiberi</i> , <i>R. solani</i> , and <i>S. rolfii</i>	[137,150, 152,153]
Carboxyacetyl chitosans	<i>B. cinerea</i> , <i>P. debaryanum</i> , and <i>R. solani</i>	[55]
Carboxymethyl chitosans	<i>A. solani</i> , <i>C. gloeosporioides</i> , <i>F. oxysporum</i> , <i>P. asparagi</i> , and <i>P. zingiberi</i>	[154]
Chitosan-metal complexes	<i>A. alternata</i> , <i>B. cinerea</i> , <i>F. oxysporum</i> , and <i>P. debaryanum</i>	[155–157]
Dithiocarbamate chitosans	<i>A. porri</i> and <i>F. oxysporum</i>	[146]
Hydroxypropyl chitosans	<i>Alternaria mali</i> , <i>Coniella diplodiella</i> , <i>Fusarium oxysporum</i> , and <i>P. piricola</i>	[158]
N,O-acetyl chitosans	<i>B. cinerea</i> and <i>P. oryzae</i>	[47,48]
N-alkyl chitosans	<i>B. cinerea</i> , <i>F. oxysporum</i> , and <i>P. debaryanum</i>	[56,57,144]
N-benzyl chitosans	<i>A. alternata</i> , <i>B. cinerea</i> , <i>B. theobromae</i> , <i>F. oxysporum</i> , <i>P. infestans</i> , <i>P. oryzae</i> , and <i>P. debaryanum</i>	[56,57,61,83, 143,144,159]
N-benzylphosphoryl chitosans	<i>B. cinerea</i>	[160]
N-cinnamyl chitosans	<i>A. alternata</i> , <i>B. cinerea</i> , <i>B. theobromae</i> , <i>F. oxysporum</i> , <i>F. solani</i> , <i>P. infestans</i> , and <i>P. debaryanum</i>	[83]
N-heterocyclic chitosans	<i>F. oxysporum</i> , <i>P. oryzae</i> , and <i>P. debaryanum</i>	[54]
N-succinoyl chitosans	<i>F. oxysporum</i>	[145]
O-acetyl chitosans	<i>A. alternata</i> , <i>B. cinerea</i> , <i>F. oxysporum</i> , <i>P. oryzae</i> , and <i>S. sclerotiorum</i>	[82,161,162]
O-succinyl chitosans	<i>A. alternata</i> , <i>B. cinerea</i> , and <i>F. oxysporum</i>	[84]
Quaternary ammonium chitosans	<i>B. cinerea</i> , <i>B. theobromae</i> , <i>F. oxysporum</i> , <i>P. infestans</i> , and <i>P. debaryanum</i>	[59–61, 143,163]
Schiff bases of chitosans	<i>A. niger</i> , <i>A. flavus</i> , <i>A. solani</i> , <i>B. cinerea</i> , <i>C. lagenarium</i> , <i>F. oxysporum</i> , and <i>Valsa mali</i>	[67,143, 164,165]
Sulfate, sulfanilamide, and sulfonilide chitosans	<i>A. solani</i> , <i>F. oxysporum</i> , <i>C. gloeosporioides</i> , <i>P. asparagi</i> , and <i>P. zingiberi</i>	[154,166]
Thiosemicarbazone chitosans	<i>A. solani</i> , <i>R. solani</i> , <i>S. solani</i> , and <i>P. asparagi</i>	[167]

Grafting alkyl or aryl moieties into chitosan molecules effectively enhanced the antimicrobial activity of the chitosan against phytopathogenic fungi [54,56,57,83,143–145]. *N*,*O*-(*p*-chlorobutyl) chitosan, *N*,*O*-decanoyl chitosan, *N*,*O*-cinnamoyl chitosan and *N*,*O*-(*p*-methoxybenzoyl) chitosan were the most potent within 18 derivatives of *N*,*O*-acyl chitosans [47] against *B. cinerea* (EC_{50} = 430, 440, 450, and 500 mg/L, respectively) and were 12- to 13-fold more active than chitosan. However, *N*-(benzo[d][1,3]dioxol-5-ylmethyl) chitosan and *N*-(methyl-4H-chromen-4-one) chitosan were the most active within five heterocyclic chitosan derivatives against *Pythium debaryanum* and *F. oxysporum* [54]. In addition, *N*-(*o,p*-diethoxybenzyl) chitosan was highest in activity within seven *N*-(benzyl) chitosan derivatives with EC_{50} 400 and 468 mg/L against *F. oxysporum* and *P. debaryanum*, respectively, but *N*-(*o,o*-dichlorobenzyl) chitosan was the most active against *B. cinerea* with EC_{50} 520 mg/L [144]. The antifungal activity of seven *N*-(cinnamyl) chitosan derivatives was also tested against *A. alternata*, *B. cinerea*, *Botryodiplodia theobromae*, *F. oxysporum*, *Fusarium solani*, *P. debaryanum*, and *P. infestans* [83]. The data proved that the presence of *N*-(*o*-methoxycinnamyl) substituted on chitosan backbone showed the highest activity among the tested compounds against the airborne fungi *A. alternata*, *B. cinerea*, *B. theobromae*, and *P. infestans* (EC_{50} = 672, 796, 980, and 636 mg/L, respectively), whereas *N*-(*p*-*N*-dimethylaminocinnamyl) chitosan was the most active against the soil-borne fungi *F. oxysporum*, *F. solani*, and *P. debaryanum* (EC_{50} = 411, 566, and 404 mg/L, respectively). Qin and coworkers [146] reported that both ammonium dithiocarbamate chitosan and triethylenediamine dithiocarbamate chitosan enhanced inhibitory effects on *F. oxysporum* and *Alternaria porri*. The activity was affected by concentration of the derivatives and fungal species. In this study, *F. oxysporum* was more sensitive than *A. porri* to chitosan derivatives. Diethyl dithiocarbamate chitosan (EtDTCCS) was also investigated against *A. porri*, *Gloeosporium theae-sinensis*, and *Stemphylium solani* at 250, 500, and 1000 mg/L, respectively [147]. Compared with chitosan, EtDTCCS showed an inhibitory index by 93.2% on *G. theae-sinensis* at 1000 mg/L, even stronger than polyoxin (82.5%). (1,2,3-triazol-4-yl) methyl nicotinate chitosan was also prepared and tested at concentrations ranging from 250 mg/L to 1000 mg/L against *R. solani*, *S. solani*, and *A. porri* [148]. It was shown that the product inhibited 94.2% of the growth of *A. porri* at 1000 mg/L, while the fungicide triadimefon had lower inhibitory index (62.2%) at the same dose. Recently, the antifungal activity of a novel class of α -aminophosphonate chitosan derivatives, (4-tolyloxy)-pyrimidyl-dimethyl- α -aminophosphonate chitosan

(α -ATPMCS), and (4-tolyloxy)-pyrimidyl-diethyl- α -aminophosphonate chitosan (α -ATPECS) was investigated against crop-threatening pathogenic fungi *Phomopsis asparagi* and *F. oxysporum* [149]. At 250 mg/L, both α -ATPMCS and α -ATPECS even inhibited growth of *P. asparagi* and *F. oxysporum* by 100%, which was even stronger than polyoxin whose antifungal index was 37.2 and 32.1%, respectively. Elkholy and coworkers [150] added that three acyl chitosan derivatives with different side chains had a significant inhibiting effect on the different stages of development on the germination of conidia or sclerotia of the sugar beet pathogens: *R. solani* and *S. rolfii*.

Three water-soluble chitosan derivatives including 1,3,4-thiadiazole (TPCTS), 2-methyl-1,3,4-thiadiazole (MTPCTS), and 2-phenyl-1,3,4-thiadiazole (PTPCTS) were synthesized and evaluated against *Colletotrichum lagenarium*, *P. asparagi*, and *Monilinia fructicola* [66]. Of all the synthesized chitosan derivatives, MTPCTS was the most effective compound with inhibitory indices of 75.3, 82.5, and 65.8% against *C. lagenarium*, *P. asparagi*, and *M. fructicola*, respectively at 1000 mg/L. The authors demonstrated that the hydrophobic moiety (alkyl or phenyl) and the length of the alkyl substituent in thiadiazole tend to affect the antifungal activity of these derivatives. Thiosemicarbazone chitosan derivatives, prepared by Qin and coworkers [167], greatly inhibited *S. solani*, *R. solani*, *A. solani*, and *P. asparagi*, and the highest antifungal index reached 100%. *S. solani* and *R. solani* were more sensitive than *A. solani* and *P. asparagi* toward chitosan derivatives.

Quaternary ammonium chitosans, a kind of chitosan derivatives with permanent cationic charges on the polysaccharide backbone, have attracted much attention to be used as antimicrobial agents [168]. That is because chitosan modified with quaternary ammonium salts becomes water-soluble and exhibits better antimicrobial activity than unmodified chitosan [169]. *N,N,N*-(dimethylalkyl) chitosans as quaternary ammonium chitosans were prepared and tested against *B. cinerea*, *F. oxysporum*, and *P. debaryanum* [59]. The results proved that *N,N,N*-(dimethyloctyl) chitosan exerted the highest mycelial growth inhibition on *B. cinerea*, *F. oxysporum*, and *P. debaryanum* (EC_{50} = 383, 812, and 440 mg/L, respectively). Moreover, spore germination of *B. cinerea* and *F. oxysporum* was greatly affected by all the compounds tested (six derivatives of *N,N,N*-(dimethylalkyl) chitosan) at concentrations ranging from 50 mg/L to 1000 mg/L and the inhibitory effect increased with increases in chain length of the alkyl substituent (methyl, butyl, pentyl, hexyl, heptyl, and octyl). Recently, a series of *N*-(aryl) and quaternary *N*-(aryl) chitosan derivatives were evaluated by *in vitro* and *in vivo* assays

against *B. cinerea* [60]. In an *in vitro* experiment, all quaternized chitosans were more active than *N*-(aryl) chitosan derivatives, and *N,N,N*-(diethylcinnamyl) chitosan was the most potent in inhibiting fungal mycelial growth (EC_{50} 1147 mg/L); however, *N,N,N*-(diethyl-*p*-dimethylaminobenzyl) chitosan was the most active (EC_{50} 334 mg/L) against spore germination. In the *in vivo* experiment, by application of compounds on tomato plants prior to inoculation with fungal spores, no disease incidence was observed with both compounds at 1000 mg/L. In this experiment, spray liquid chitosans improved total phenolics content and guaiacol peroxidase in tomato leaves compared with the control. In another study, these derivatives exhibited high activity against mycelial growth of *B. cinerea*, *B. theobromae*, *F. oxysporum*, and *P. infestans* and inhibited, *in vivo*, exocellular enzymes including polygalacturonase, pectin-lyase, polyphenol oxidase, and cellulase at a concentration of 1000 mg/L [61]. The results demonstrated that the grafting of aryl moiety onto chitosan molecule following quaternization of the derivatives successfully inhibited microbial growth.

Guo and coworkers [143] added that the Schiff bases of phenyl and hydroxyphenyl chitosans and their *N*-substituted derivatives had a slight activity against *B. cinerea*, and the inhibitory indexes were 26.8, 33.5, 39.3, and 42.3% at 1000 mg/L, respectively, compared with 45.4% for chitosan. However, quaternized chitosan derivatives have better antifungal activity against *B. cinerea* than unmodified chitosan, Schiff bases of chitosan, and *N*-substituted chitosan derivatives, and the inhibitory indexes of quaternary phenyl chitosan or quaternary hydroxyphenyl chitosan were 81.2 and 58.6%, respectively, at 1000 mg/L. Similar to the antifungal activity against *B. cinerea*, the quaternized chitosan derivatives have better activity than those of chitosan, Schiff bases of chitosan, and *N*-substituted chitosan derivatives against *C. lagenarium*. The inhibitory indexes were 71.5 and 55.8% for quaternary phenyl and hydroxyphenyl chitosans, respectively, at 1000 mg/L. In another study [170] it was found that four quaternized chitosans: *N*-(2-hydroxyl-phenyl)-*N,N*-dimethyl (NHPDCS); *N*-(5-chloro-2-hydroxyl-phenyl)-*N,N*-dimethyl (NCHPDCS); *N*-(2-hydroxyl-5-nitro-phenyl)-*N,N*-dimethyl (NHNPDCS); and *N*-(5-bromic-2-hydroxyl-phenyl)-*N,N*-dimethyl (NBHPDCS) showed higher antifungal activity against *B. cinerea* and *C. lagenarium* than chitosan, and the inhibitory indexes at 1000 mg/L, for *B. cinerea* were 58.6, 86.7, 68.8, and 66.6%, respectively, while for *C. lagenarium*, they were of 55.8, 72.6, 63.6, and 79.5%, respectively. Three other quaternary chitosan derivatives were prepared by Li et al. [171] by reaction of chloroacetyl chitosan (CACS) with pyridine

(PACS), 4-(5-chloro-2-hydroxybenzylideneamino)-pyridine (CHPACS), and 4-(5-bromo-2-hydroxybenzylideneamino)-pyridine (BHPACS) and tested against *Cladosporium cucumerinum*, *M. fructicola*, *C. lagenarium*, and *F. oxysporum*. CHPACS and BHPACS were two effective antifungal derivatives of chitosan and the inhibitory indexes against *C. cucumerinum*, *M. fructicola*, *C. lagenarium*, and *F. oxysporum* ranged from 45.3% to 100% at 500 mg/L and from 71% to 100% at 1000 mg/L. The antifungal activity of chitosan can be improved by increasing the DS of alkyltrimethyl ammonium groups on the polymer chain. Oliveira Pedro et al. [172] reported that the inhibition indexes of propyl and pentyl (trimethylammonium) chitosan for *A. flavus* increased with the DS increase, and both derivatives exhibited inhibition values by three and six times, respectively, higher than those obtained with chitosan. Chitosan-metal complexes, with a positive charge on their structure, have also a wide spectrum of antimicrobial activity against plant pathogens. They may serve as a reservoir for the slow release of metal ions that increase the biological activity of chitosan. A strong effect was obtained with chitosan-copper (II) and chitosan-zinc (II) complexes against *Aspergillus fumigatus*, *F. solani*, and *F. graminearum* [173,174].

Effect on Viral Diseases

The antiviral activity of chitosan has been studied on plant diseases caused by viruses and viroids through enhancing the host's hypersensitive response to infection by viruses [175–179]. For example, on bean leaves, local infections produced by alfalfa mosaic virus were completely controlled by chitosan at 1000 mg/L, either sprayed or added to the inoculum [178]. Similar inhibition was also reported on tomato leaves treated with chitosan at the same concentration and inoculated with potato spindle tuber viroid [176]. In these studies, systemic resistance was induced by chitosan on various host virus combinations. Iriti et al. [180] mentioned that the treatment with chitosan at 1000 mg/L enhanced tobacco-inducible defenses against tobacco necrosis necrovirus (TNV) and significantly reduced the size of lesions. This resistance was associated with callose deposits, micro-oxidative bursts and microhypersensitive responses [180]. The network of microhypersensitive response elicited by chitosan would then activate plant defense by mimicking a localized virus infection [181]. Thus, accelerating the subsequent hypersensitive response induced by the challenge inoculation with TNV. Ultimately, this would impair virus replication and translocation, reducing the number and size of virus lesions. Similarly, chitosan with 1.5, 17, and 25% DA inhibited the formation of local necroses induced by tobacco

mosaic virus for 50–90% [182]. Chitosan with different MW (3.36×10^3 and 12×10^4 Da), at 1000 mg/L, induced resistance to viral infection in potato plants and the highest antiviral activity was obtained by chitosan of 12×10^4 Da [183]. In another experiment, infected potatoes by potato virus X after pretreatment with chitosan, showed significant less infestation, and the treated leaves accumulated less virus amount than the control [183].

Effect on Nematodes

A number of studies found that chitin and chitosan have useful nematostatic and nematicidal activity for agricultural applications by admixing effective amounts with a plant growth medium [99,184–190]. The chitosan complex also provides a source of nitrogen in slow-release form, making it particularly suitable for combination with fertilizers and soil conditioners. Chitinous amendments effectively reduced the levels of *Meloidogyne arenaria* and *Heterodera glycines* [187,189,190]. The control level by chitin products was sufficient for them to be registered and marketed as commercial nematocides such as ClandoSan® 618 [191]. The application of different MW chitosans as a soil amendment was used in controlling pine wilt disease caused by *Bursaphelenchus xylophilus* nematode in maritime pine (*Pinus pinaster*) and stone pine (*Pinus pinea*) [192]. At the end of the experimental period (24 days after inoculation), *P. pinaster* and *P. pinea* untreated plants presented 3825 ± 100 and 70 ± 47 nematodes, respectively. In *P. pinaster*, high MW chitosan induced a 21.9-fold reduction in nematodes numbers, whereas in *P. pinea* the low MW reduced nematodes numbers up to sevenfold, compared with the untreated plants [192]. The nematicidal activity of four MW chitosans (2.27×10^5 , 3.60×10^5 , 5.97×10^5 , and 9.47×10^5 Da) was assayed *in vitro* and in pot experiments against *Meloidogyne incognita* [193]. In laboratory assays, low MW chitosan (2.27×10^5 Da) was the most effective in killing nematodes (EC_{50} 283.47 and 124.90 mg/L after 24 and 48 h of the treatment, respectively). In a greenhouse bioassay, all compounds mixed with soil at one- and fivefold of the LC_{50} values significantly reduced population, egg mass, and root galling of tomato seedlings compared with the control. Chitin and chitosan were also evaluated against *M. incognita*, infecting tomato in a glasshouse [194] by mixing with soil at 1, 3, 5, and 10 g/kg, and their nematicidal activity was compared with synthetic nematocide oxamyl at 0.01 g/k¹. In this study, chitin and chitosan reduced root galls and second-stage juveniles (J_2) of *M. incognita* in the soil in a dose-dependent manner. Chitosan was more effective in reduction of galls and J_2 than chitin; however, both compounds were less than oxamyl in reducing the number

of J₂ in the soil. Recently, novel chitosan–cinnamon beads were prepared and their nematocidal activities were *in vitro* investigated against *M. incognita* [195]. Inhibitions of hatch by chitosan beads loaded with 0.5% cinnamon extract and 5% cinnamon powder were 82 and 78.8%, respectively. J₂ mortality percentages following the 5% cinnamon powder and 0.5% cinnamon extract–chitosan bead treatments were 85.0 and 95.8%, respectively, against *M. incognita* after 48 h incubation [195].

These results suggest that chitosan–cinnamon beads may be considered a drug carrier, because the nematocidal activity against *M. incognita* was prolonged.

Effect on Insects

Chitosan has a negligible insecticidal activity; however, its derivatization with toxiphor group improved the insecticidal activity against some pests [54,56,57,161,196,197]. The insecticidal activity of chitosan and oligochitosan against plant-feeding lepidopterous and homopterous has been studied by Zhang and coworkers [77]. The results indicated that the insecticidal activity of chitosan to *Plutella xylostella* was higher than that to *Spodoptera exigua* and the mortality reached up to 80%. Bioassays with 17 derivatives of *N*-(alkyl) chitosans at a rate of 5000 mg/g diet demonstrated that *N*-(propyl) chitosan, *N*-(undecanyl) chitosan, and *N*-(3-phenylpropyl) chitosan strongly inhibited larval weight gain in cotton leafworm *Spodoptera littoralis*, with reductions of 76, 66, and 65%, respectively, after 4 days of feeding [57]. In another study, *N*-(*p*-isopropylbenzyl) chitosan, *N*-(2-chloro-6-fluorobenzyl) chitosan, and *N*-(*o*-nitrobenzyl) chitosan caused 46, 100, and 46% mortality of *S. littoralis* larvae, respectively at 5000 mg/g diet [56]. Moreover, a series of *O*-(acyl) chitosan derivatives with DS between 0.02 and 0.28 showed high inhibition growth against *S. littoralis* larvae and the most active compound was *O*-(decanoyl) chitosan after 5 days of feeding [161].

Chitosan also exhibited insecticidal activity against aphids at a range of concentrations from 600 mg/L to 6000 mg/L. For example, chitosan showed mortalities of 93–99% against *Hyalopterus pruni* on flowers. It showed 60–80% mortality against *Rhopalosiphum padi*, *Metopolophium dirhodum*, and *Aphis gossypii*, while *Sitobion avenae* and *Myzus persicae* showed a lower susceptibility to chitosan [77]. Four MW chitosans (2.27×10^5 , 3.60×10^5 , 5.97×10^5 , and 9.47×10^5 Da) were also active against oleander aphid *Aphis nerii*, and the results indicated that 3.60×10^5 and 5.97×10^5 Da chitosans were the highest in aphicidal activity by leaf-dip bioassay after 24 h of treatment (48 and 49% mortality, respectively, at 1000 mg/L) [196].

Aphicidal activities of chitosan diethyl phosphate and chitosan ethyl carbamate at different concentrations have been evaluated against green peach aphid (*M. persicae*) and compared with imidacloprid [197]. Chitosan N-diethyl phosphate and chitosan ethyl carbamate at 5000 mg/L showed the highest lethal activity in the pest insects *Elasmopalpus angustellus*, *Helicoverpa zea*, and *Liriomyza huidobrensis*. The percentage of mortality in these pest insects increased with the addition of diethyl phosphate and ethoxycarbonyl groups to chitosan. A systemic effect was not observed using chitosan and chitosan carbamate, suggesting that the insecticidal activity was due to the hydrolysis of the diethyl phosphate moiety [197–200].

TECHNOLOGICAL APPLICATIONS OF CHITOSAN IN AGRICULTURAL DISEASES AND PEST CONTROL

Chitosan Applications in Seed-Coating Technology

Plant seeds suffer from attacks by many pests and diseases that result in significant decrease in crop yield throughout the world. To prevent considerable economic losses, many studies have been carried out on seed-coating technology [201,202]. Chitosan is a promising candidate as a seed-coating agent to protect them from different microorganisms such as bacteria, yeast, fungi, and pest insects [203–210]. The soil-borne fungi *F. solani* and *Colletotrichum lindemuthianum* were inhibited by seed treatment with chitosan and N-(carboxymethyl) chitosan [104,108,211]. Benhamou and coworkers [110] reported that chitosan at concentrations of 500–1000 mg/L reduced disease incidence caused by *F. solani* in tomato plants, when used as seed coating or admixing with soil. Although chitosan at 1000 mg/L delayed disease development, emergence of wilting symptoms appeared 7–10 days after inoculation, while an 80% death of the plants was observed one week later.

Seeds treatment by chitosan improved germination of several crops and increased their yield. For example, treatment of wheat seeds with chitosan applied for controlling strawbreaker disease (*Pseudocercospora herpotrichoides*) had a positive effect and kept the wheat upright throughout the harvest period [212]. Zeng and Luo [213] reported that wheat seeds coated with chitosan (2000–8000 mg/L) significantly improved germination rate, seedling growth parameters, and physiological indexes (superoxide dismutase, peroxidase, and catalase). A chitosan of 65% DA showed good antifungal effect and ability to promote wheat resistance to infection by *Bipolaris sorokiniana* when used as seed coating [92,93]. It was also reported that chitosan, at 2000–8000 mg/L, increased wheat seed resistance to seed-borne

F. graminearum infection and improved their germination [214,215]. Similarly, peanut seeds soaked in 7500 mg/L of chitosan solution were reported to increase germination and energy, lipase activity, gibberellic acid, and indole acetic acid levels by 14.4, 4.5, 80, and 60.3%, respectively [216]. A cucumber seed-coating agent prepared from a natural polysaccharide (MW 3×10^4 to 14×10^5 Da and DDA 80%), a fertilizer and a microelement, indicated high protection effect against insect pests and increased yield by 8.5–9.3%, while the material cost was decreased by 16.7% compared with traditional agents [217].

Chitosan was also studied as a coating agent for soybean seeds to control *Agrotis ipsilon*, soybean pod borer (*Maruca vitrata*), and aphid [209]. The results indicated that chitosan coatings had antifeeding effect against the tested pests; with a concentration increase, the antifeedant rate was significantly increased, especially when in the concentration of 50 g/L, antifeedant rate of *A. ipsilon*, soybean pod borer, and aphid reached 82.89, 87.24, and 80.21%, respectively.

Chitosan applications in rice production, through seed soaking and application to soil four times throughout the cropping season, showed an ability to control diseases and significantly increased the yield over other treatments [102]. Zeng and Shi [218] performed a new type of chitosan with some additives as a seed-coating agent for rice. By using this formulation, safer, cheaper, and more environmentally friendly, the antifungal efficiency increased as dose increased. The 1:20 concentration was the best for inhibiting growth of *R. solani* and *Fusarium moniliforme*. Ruan and Xue [219] mentioned that rice seed coating with chitosan accelerated germination and improved tolerance to pests. Chitosan also improved the vigor of maize seedlings after coating [220,221]. In this study, chitosan reduced malonyldialdehyde content, altered the relative permeability of the plasma membrane, and increased soluble sugars, proline, peroxidase, and catalase activities. Lizárraga-Paulín and coworkers [222] reported that maize seed coating with chitosan (20 g/L) increased germination and protein content in two different maize varieties, while there was no significant effect on the catalase activity of treated seeds and seedlings. It was also used as a seed-coating agent to protect cotton from *A. gossypii* and *Helicoverpa armigera*. It enhanced germination, plant growth, and lint yield [223].

Chitosan Applications in Postharvest Technology

In recent years, interest in chitosan edible films and coatings for agricultural commodities such as fruits and vegetables increased and developed mainly

due to concerns over the disposal of conventional synthetic plastic materials [224–238]. These biofilms have a homogeneous matrix, stable structure, good permeability, good water barrier, and mechanical properties [228,239–247]. Chitosan coatings are designed to slow down the antimicrobial release from the surface; thus, smaller amounts of antimicrobials would be needed. They have been used on fruits and vegetables with high antimicrobial activity against several pathogens [15,111,225,248–255]. El-Ghaouth and co-workers [112] found that coatings of strawberry, bell pepper, and cucumber by chitosan, reduced lesion occurred by *B. cinerea* and *R. stolonifer*, as well as delayed ripening. Chitosan coating significantly delayed the development of brown rot disease caused by *M. fructicola* on peach and litchi fruit [256–259]. Gray mold and blue mold, caused by *B. cinerea* and *P. expansum*, respectively, of sweet cherry and tomato fruit were reduced by dipping them in chitosan solution; however, the treatments were more effective against the gray mold than the blue mold [23,237]. Our study confirmed that different MW of chitosan reduced fungal decay by *B. cinerea* on tomato fruits, and all chitosans at concentrations of 2000 mg/L and 4000 mg/L, exhibited complete fungal control in inoculated fruits [225]. Coating citrus fruits with chitosan was effective in controlling decay caused by *P. digitatum* [255,260–262].

It was observed that chitosan has also the ability to elicit defense-related enzymes such as peroxidase, PAL, chitinase, and β -1,3-glucanase or pertinent substance in treated fruits and vegetables. For example, PAL activity (a key enzyme for synthesis of phenolic compounds, usually characterized by antimicrobial activity) was increased in grapes inoculated with *B. cinerea* and coated with chitosan [138,253]. A synthesis of anthocyanin was decreased in strawberries infected with *B. cinerea* and *R. stolonifer* and coated with 10 g/L chitosan [258,263]. Similar results were observed for other fruits and vegetables such as citrus [226,255,262], mango [264], sweet cherry fruit [23], tomato [136,225], strawberries and raspberries [136,265,266], Arabidopsis [267], and papaya fruit [268] when they were inoculated and coated with chitosan. Moreover, oligochitosan has been effective in inducing the production of nitric oxide and hydrogen peroxide in *Brassica napus* [269] to elicit PAL and peroxidase activities, lignin deposition in wheat leaves [270] and citrus fruit [255], and increases the activities of PAL, chitinase, transcription of defense-related genes β -1,3-glucanase and accumulation of pathogen-related protein in rice cells in suspension culture, and also in rice seedlings [271]. Therefore, it can be proved that the inhibitory effect of chitosan coating originates from a combination of its antimicrobial properties and ability to stimulate defense responses in the host against microorganism infection.

Chitosan Applications in Controlled-Release Agrochemicals

Concerns about the environment have arisen with the excessive application of agrochemicals such as fertilizers and pesticides. Slow-release technology improves delivery of pesticides by optimizing pesticide activity profile while lowering their losses and contact with the environment [272–274]. Controlled release formulations become interesting in cases of antagonist activity of biocides. The formulation in this case should be programmed to release each compound at different times [275,276]. It is important to use compatible, environmentally friendly, biodegradable, nontoxic products and less expensive materials in a commercial formulation for practical field applications. The biopolymers cellulose, agarose, dextran, alginates, carrageenans, starch, and chitosan and proteins including gelatin and albumin are interesting materials in controlled-release systems.

A new type of chitosan-coated nitrogen, phosphorus, and potassium formulation to improve the utilization of these fertilizers and water resource at the same time was prepared. Its core was water-soluble, granular fertilizer composed by an inner coating of chitosan and an outer coating with an absorbent polymer [277]. Chitosan beads have been extensively used as devices for controlling release of many substances such as pesticides in pest control. Active compounds that have been encapsulated include, for example, atrazine, methyl parathion, trifluralin, chlorpyrifos, thiocarbamates, and *Bacillus thuringiensis israelensis* (Bti) toxins [278–280]. Teixeira and coworkers [278] found a controlled-release effect by coating urea and the herbicide atrazine pellets with films of *N*-acetyl or *N*-propionyl chitosan. The data showed that compared with uncoated beads, the release period extended by a factor of 50 for atrazine beads and a factor of 180 for urea beads. The release time of atrazine was increased to six months in *N*-acetyl chitosan beads and one year in *N*-propionyl chitosan beads. Blending of chitosan with other biopolymers such as alginate, gum, and starch is a convenient method to improve its properties for practical applications [281,282]. Chitosan/alginate beads are used as matrices for slow release of pesticides in agricultural applications [283–285]. A pesticide formulation based on chitosan and cashew gum beads containing insecticide dichlorvos [2,2-dichlorovinyl dimethyl phosphate (DDVP)] was prepared and tested as a larvicide for controlling *Aedes aegypti* larvae [281]. *In vivo* release showed that chitosan/cashew gum beads reached a plateau after 30 h resulting in high larval mortality (60%), whereas chitosan beads caused only 42% mortality after 30 h and seemed not to have released all larvicide content up to 72 h. The persistence studies revealed that the DDVP-loaded beads were effective up to 336 h. The

herbicide imazaquin was also encapsulated with chitosan/starch beads and reinforced with alginate [272]. Chitosan nanoparticles incorporated insecticidal protein beauvericin (CSNp-BV) was prepared by ionic gelation method to improve insecticidal activity against *Spodoptera litura* [286]. Loading efficiency and entrapment efficiency was found to be 82 and 85%. The *in vitro* drug release profile showed accumulative release that reached almost 91% after 24 h. Pesticidal activity revealed that all life stages were susceptible to the CSNp-BV formulation and the maximum mortality was recorded in early larval instars. CSNp-BV treatment reduced pupal and adult emergence.

Nanoparticles composed of chitosan and sodium tripolyphosphate (TPP) were also prepared to produce an efficient herbicidal formulation for safe controlling of weeds in cultivations of maize and mustard [287]. The efficiency of nanoparticles in association with paraquat was 62.6%. Encapsulation of the herbicide controlled its diffusion, release, and sorption in soil. The nanoencapsulated herbicide was less toxic by cytotoxicity and genotoxicity assays than the pure compound, indicating its potential herbicidal activity while at the same time reducing environmental hazards. Carboxymethyl chitosan carrying ricinoleic as an emulsifier was used to encapsulate the insecticide azadirachtin nanoparticles [288]. Nanoparticles of 200–500 nm were obtained with loading azadirachtin higher than 50% and tested for their release in outdoor condition. After five days of sun exposure, all content of control samples were lost, while the nanoparticles had a constant residual concentration during 12 days of the experiment [288]. The insecticide etofenprox was encapsulated using a nanosized chitosan carrier in three types according to a difference in release patterns by adjusting the molecular weight and concentration of chitosan [289]. Release properties of etofenprox and its biological activity against *S. litura* suggested that such controlled-release formulation is used as a method for preventing loss of etofenprox, increasing its activity against the target pest.

The volatile essential oils show promising activity against agricultural pests in different areas such as insect repelling and fumigation in stored products [290,291]. However, the release speeds of these oils are usually high and affected by the environment conditions [292]. Therefore, utilizing encapsulation technology to achieve the goal of constant release is one of the most effective methods [293]. Chitosan has been examined in the development of essential oils delivery systems [294–296]. The results indicated that chitosan possesses a heat-shrinking property, thus the change in pore space between chitosan and the cell wall membrane molecules led to slow release of the essential oil from the formulation [297,298].

PROPOSED MECHANISMS OF CHITOSAN ACTION AGAINST PLANT PESTS AND DISEASES

Many studies have been carried out on the mode of action of chitosan against microbial plant diseases and other pests. In antimicrobial action, it is known that the chitosan acts at extracellular (plasma membrane) and intracellular levels (molecule penetration into the microbial cell) [107,299–302]. The most common mechanism of action is that the positively charged chitosan gives this one great physiological and biological polymer property. The cationic nature of chitosan leads to binding with sialic acid in phospholipids, which consequently modifies the cell permeability and causes materials prevented from entering the cell and/or material being leaked from the cell [9,10,65,68,303–309]. El Ghaouth and coworkers [111] confirmed that chitosan provoked the leakage of amino acids and proteins of *R. stolonifer* cells. Similar results were obtained on three isolates of *R. stolonifer*, whereby chitosan-increased release of proteins and glucose consumption [310]. In other studies, potassium ion leakage was demonstrated by effect of chitosan on fungal cells, being more pronounced for the first 5 min [311,312]. In addition, chitosan caused a decrease in the H^+ -ATPase activity on plasma membrane of *R. stolonifer*; this effect could provoke the accumulation of protons inside the cell, which would result in the inhibition of the chemiosmotic-driven transport that allows the H^+/K^+ exchange [311]. Another important mechanism involves penetration of the chitosan oligomer into the cells of microorganisms and inhibits the growth of cells by preventing the transformation of DNA into RNA [9,11,307,312–315]. It was found that chitosan quickly penetrated the conidia of *F. oxysporum* and caused ultrastructural alterations in the treated spores [301]. Xu and coworkers [118] reported that oligochitosan penetrated the fungal cell of *P. capsici* and caused disruption on the endomembrane system, such as distortion and disruption of most vacuoles, thickening of plasmalemma, and appearance of unique tubular materials. Chitosan also acts as a chelating agent that selectively binds trace metals and thus inhibits toxin production and microbial growth [211].

Possible mechanisms to suppress viral plant infections by chitosan are also proposed; however, the mechanism is not well understood. The ability of suppressing viral infections does not depend on the type of virus; because chitosan affects the plant itself by induction of a broad spectrum of protective reactions, limiting systemic spread of viruses and viroids over the plant and leading to the development of systemic acquired resistance [183,316–319]. To date, various studies involving factors that are involved in determining the antiviral activity of chitosan and chitooligosaccharides were

performed. However, one possible explanation is that the cationic charges of amino groups and increases DA of chitosan and chitooligosaccharides activate the immune and defense systems in plants [43,316]. Kochkina and Chirkov [320] studied the effect of chitosan fragments with different DPs and some chemical chitosan derivatives on the infection of *B. thuringiensis* by phage 1-97A. They have shown that chitosan inhibited phage infection and inactivated phage particles. The extent of inhibition of phage infection inversely depended on the DP of chitosan fragments. In contrast, low MW chitosan with higher DP was more active in inhibiting coliphage infection than that of low MW with lower DP [321]. Therefore, it can be expected that chitosan can be involved in the inhibition of the replication of bacteriophages by different mechanisms.

The mechanisms of action of chitin and chitosan on plant parasitic nematodes remain unclear. However, one proposal is that they control nematodes by indirect action as promoting the growth of beneficial chitinolytic microorganisms in soil that parasitized the eggs of the nematodes [190,322]. Duncan [323] and Stirling [324] concluded that there was insufficient evidence to support this mode of action without detectable egg parasitism by chitinolytic fungal when chitin was applied to the soil. Therefore, another mechanism has been suggested, whereby chitin breaks down in the soil and releases ammonia having nematicidal action. This hypothesis represents a more direct nematicidal action of chitin and chitosan [189]. However, control of nematode populations by addition of chitin has also been found over longer periods than would be expected from short-term release of ammonia that dissipates quickly [322], thus indicating that another mechanism may be operating in chitin and chitosan amended in soil.

The proposed mechanisms of chitosan action as seed coating depend on the chitosan molecule stimulating the plant to produce antibodies, which causes a repellent effect against different pests [208,325–327]. Chitosan has excellent film-forming properties, making it easy to form a semipermeable film on the seed surface, which maintains the seed moisture, and thus promotes germination. The initial antimicrobial properties of chitosan are at the seed surface; therefore, chitosan must penetrate the seed coat and cell membranes before exerting its influence on the cellular metabolism of the plant. Studies using radiolabeled chitosan indicated that the chitosan applied to the seed was detected in other parts of the seedlings as they develop. Lignin accumulation following wheat seed treatments rose significantly above levels in the untreated tissue and may have contribute to stem strength [204,205,325]. Furthermore, chitosan film has a good selective permeability,

which can prevent penetration of oxygen to the film inside, restricting the loss of CO₂ so as to adjust the seed respiration and thus decrease the consumption of internal nutrients [328]. Chitosan can also increase the soluble sugars and strengthen the protease conversion activity in the free protein, increasing free amino acids content, which has obvious inhibitory effect for many plant pathogens [19,221,222,329].

CONCLUDING REMARKS

Naturally occurring products with interesting biological activity against agricultural diseases and insect pests have been receiving more attention in recent years as alternatives to harmful synthetic pesticides. Among these natural compounds, the biopolymer chitosan has been considered as an antimicrobial and pest-controlling agent in agricultural application. This molecule can be used in a number of ways to reduce plant disease levels and prevent the development and spread of diseases. The biological activity of chitosan on plant microorganisms and pests comes from its direct action and elicitation of defense-related enzymes. The activity depends on several factors such as type of chitosan, MW, DDA, solubility, positive charge density, molecular modification, pH, concentration, capacity of chelation, and the type of target organism. Although many studies focus on plant protection, many explanations still remain to be studied. Finally, studying the incorporation of chitosan products into IPM programs remains to be pursued in several crops, requiring deeper investigation of the modes of action of these products.

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CHAPTER 8

Prospects for the Use of Chitosan and Other Alternatives in Ornamental Conservation

Laura Leticia Barrera Necha, Silvia Bautista-Baños

INTRODUCTION

Ornamental plants constitute natural living beauty that enriches the quality of human life and represents an important economic grouping within the ornamental industry. Worldwide it has increased significantly in volume and value of production, as well as specialization and commercialization. To achieve success in producing ornamentals, it is necessary to produce quality, homogeneous, and standardized products, meet production volumes, and have distribution and marketing channels. Countries leading in the production of ornamentals have technology and adequate harvest and postharvest handling, high-quality standards, and good marketing. The Asia-Pacific region has more than two-thirds of the world's acreage. China with 40% and India with 15% have a majority in the world acreage of flowers and plants. Japan, Taiwan, and Thailand are other major flower-producing countries in this region. The European Union (EU) represents a 12% share of the world's area of flowers. The area of flowers in Africa is small with a 1.4% share. Kenya is the largest African grower. The European Union and Mexico are among the most important world producers. [Figure 8.1](#) shows the most important producers of flowers and plants in the world with their share of the world area of flowers and plants in the year 2004. The United States with a share of 12% of flowers and plants is one of the countries with the highest production intensity per hectare ([Figure 8.2](#)). Countries with a high ratio of protected production areas such as Denmark, Sweden, and the Netherlands achieve the highest yields per hectare. We can conclude that the production of flowers and plants under protection is much more intensive and effective than production in open areas.

The demand for ornamentals in the international market has increased in recent years, mainly in countries with high purchasing power, such as Germany, England, Netherlands, and France. The value of exports of ornamental flowers and plants from the European Union in 2004 reached

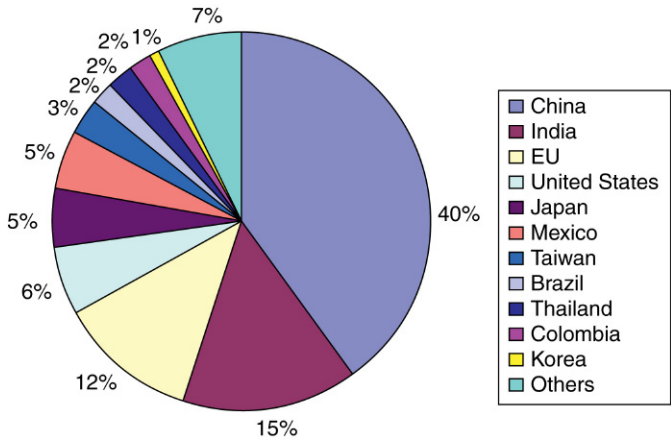


Figure 8.1 *Global Area of Production of Flowers and Plants. European Commission [1].*

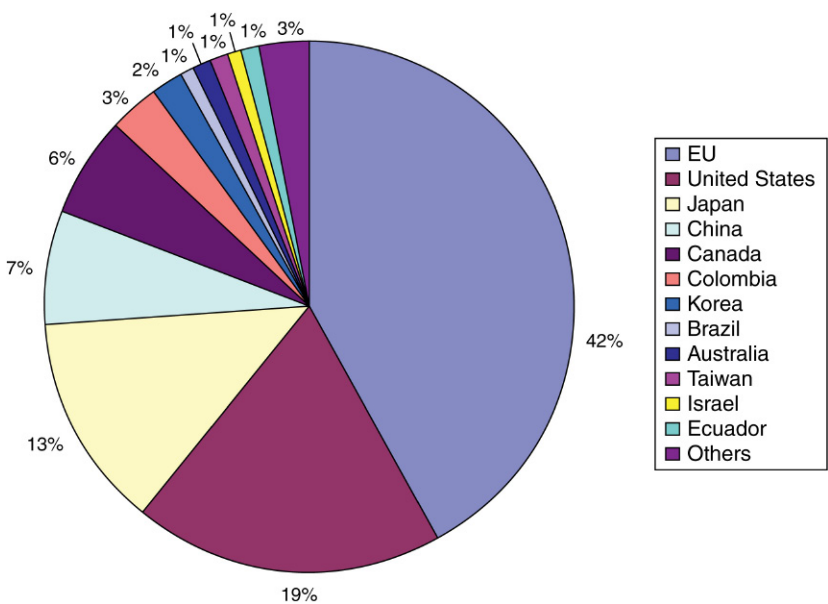


Figure 8.2 *Global Production of Flowers and Plants. European Commission [1].*

the level of \$446 million. The most important European exporters are the Netherlands with an 86.9% share of total exports. The second is Italy with a share of 5.2%, and the third is Germany with 4.5%. France is in fourth position at 1.6%. The main EU export destination for ornamental flowers and plants is the United States, representing 24% of total EU exports. Others destinations are Russia 23%, Switzerland 23%, Norway 6%, and Japan 3%

[2]. Cash receipts of the floriculture and nursery industries combined were estimated at \$14.3 billion in 2003 in the United States [3]. Colombia exported 2% of the world total production and their main markets are United States, Netherlands, and Russia. In Mexico during the period 2000–2011, the value of floriculture production increased at an average annual rate of 9.5%, reaching a value of \$384 million [4].

COMMON PHYTHOPATHOGENS OF ORNAMENTALS

The ornamental industry has continued to be rocked by disease outbreaks. A few bacteria (*Xanthomonas* and *Erwinia*) are major pathogens of ornamental crops [5–7] that are afflicted both pre- and postharvest by many diseases causing fungi. Pathogens cause root rot *Phytophthora*, *Pythium*, and *Rhizoctonia* [8,9]. The most common *Fusarium* wilts in greenhouses appear on *Dianthus caryophyllus* (carnation) (*Fusarium oxysporum* f. sp. *dianthi*), *Gladiolus* spp. (gladiolus) (*F. oxysporum* f. sp. *gladioli*), *Cyclamen* spp. (cyclamen) (*F. oxysporum* f. sp. *cyclaminis*), and *Chrysanthemum grandiflora* (chrysanthemum) (*F. oxysporum* f. sp. *chrysanthemi*). Wilt, yellowing, chlorosis, drooping (mostly of the lower leaves), stunting, and brown discoloration of the vascular bands up to the top of the stem are the dominant symptoms. Wilting of the lateral shoots and large lesions on the lower part of the stem are also common in *Fusarium* wilt of carnation. All these *F. oxysporum* formae have more than one race. Each of them infects one host but may colonize the root system of other plants as well. They survive in the soil for several years, due to the production of thick-walled chlamydospores, but inoculum is reduced over the years. *Fusarium* wilt in carnation, cyclamen, chrysanthemum, and gladiolus is favored by higher temperatures [9–12]. *Alternaria* leaf spot of carnation (*Alternaria dianthi*) mostly infects carnation cuttings during mist propagation and in wet parts of greenhouses. Small purple spots on the leaves are the first symptoms. Soon they enlarge, and their center turns brown and then black due to the masses of spores that develop. Stem infection usually appears on the knots [13]. *Botrytis tulipae* causes tulip fire blight. Spots of various types on leaves and flowers, lesions on the stem, blossom blight, and bulb rot are the dominant characteristics. *Botrytis gladiolorum* damages gladiolus and some other Iridaceae. Large spots on leaves and the stem, pinpoint spots on the flowers, neck rot, and soft rot of corms are the most common symptoms. *Botrytis* spp. also infects all types of propagating material, which are either destroyed before planting out or become weak plants that may die before or after emergence. Finally, it may cause severe postharvest

losses in plant products during storage or transportation [14,15]. Increasing worldwide demand for high-quality floricultural produce requires attention by the industry to postharvest disease management. However, the use of synthetic fungicides is becoming increasingly problematical. Their overuse has led to proliferation of fungicide resistance within pathogen populations, and they are becoming more unacceptable due to attendant human and environmental health risks. Thus, it is necessary to explore alternatives for controlling pre- and postharvest diseases in ornamental plants. Among those alternatives, the use of naturally occurring compounds such as chitosan, plant extracts, and essential oils has been explored.

Plant health management programs for ornamentals, as for other crops, must ensure the health of the plant material entering production, provide cultural conditions that work against disease development, and make arrangements to correctly identify and treat problems that do arise. The integration of these principles is the secret of effective management. Nowadays, advancements in scientific knowledge of plant health management for ornamentals relate to the key principles of sanitation, host resistance, bio-control, chemical control, and alternatives under experimentation (chitosan, extracts, and essential oils).

PHYSICAL ALTERNATIVES

Sanitation

The extensive adoption of soilless medium has eliminated many disease problems caused by soil-borne plant pathogens in ornamentals production. Soil is still used as a potting mix component or in ground beds. The accepted treatment for freeing soil from pathogens since the 1960s has been the use of aerated steam for pasteurization [16–18], which leaves some beneficial microorganisms in the soil rather than creating a biological vacuum. Structural solarization recently has been developed for greenhouse use [19]. Anand and Gautam [20] reported the use of soil solarization, fungicide gladiolus corm dip, and soil amendments for *Fusarium* wilt. Corms were treated with carbendazim, carbendazim + mancozeb (0.2%) or *Trichoderma viride* formulation (0.5%) before sowing. Three fungicide drenches at 10-day intervals for a month after sowing of corms were found quite effective. Soil amendments with cabbage leaf residue together with soil solarization were found to be the most effective treatment for disease control (98.5%).

Water used for irrigation can also be a source of pathogens or can provide a medium by which pathogens are introduced to plants. The technology

for water disinfection by physical, chemical, and biological means is critically important for greenhouse and nurseries using recirculating irrigation [21,22].

Thermotherapy

A key aspect of plant health management is starting with clean propagative material. Most ornamentals are multiplied via vegetative propagation, which means a greater danger of pathogen passage than in seed propagated crops. Thermotherapy is sometimes employed to free plant pathogens [23]. Hot water can be used for pathogen eradication from corms, bulbs, tubers, and seeds, and has the advantage of penetrating to internally harbored pathogens. Crop sanitation includes control of weeds that are pathogen reservoirs as well as control of arthropods that vector pathogens [24]. Although short-term exposure to high temperature will often free seeds from bacteria and fungi, generally longer periods of exposure are more effective for eliminating viruses [25]. Aerated steam used at 56–57°C for 30 min is safer than hot water and more effective than air for seed treatment [26]. Although short-term exposure to high temperature will often free seeds from bacteria and fungi, any heat treatment poses a risk of reduced germination percentage. As a result, this approach is not widely used by the flower seed industry.

HOST PLANT MATERIAL RESISTANCE

Development of transgenic crops holds promise in floriculture, as a large number of cut flower crops have been transformed including rose, chrysanthemum, and carnation [27]. *Rosa* spp. (rose) plants transformed with the antimicrobial protein gene, Ace-AMPI, were more resistant to *Sphaerotheca pannosa*, cause of powdery mildew, than were nontransformed plants [28]. *Chrysanthemum morifolium* Ramat. cv. Kundan transformed with the coat protein (CP) gene of cucumber mosaic virus (CMV) showed delayed resistance when challenged by CMV-chrysanthemum strain, which produced good quality blooms as compared to the susceptible ones [29]. Insertion of the chitinase transgene in roses resulted in plants more resistant to *Diplocarpon rosae*, cause of black spot disease [30]. Many studies were carried out on plant regeneration from callus as well as suspension cultures of gladiolus [31–35]. Direct DNA transfer using callus as well as cell suspension cultures through biolistics have already been reported [36–38]. The transgenic gladiolus plants thus obtained were grown in the greenhouse under federal guidelines for containment of genetically engineered plants. Transformation

of a number of carnation varieties with a gene encoding a chitinase, capable of hydrolyzing fungal cell walls *in vitro*, has been reported. CaMV35S promoter produced some events with significantly delayed onset of symptoms in a standard glasshouse trial. One line exhibiting delayed onset of symptoms and delayed time to death, following challenge with *F. oxysporum* f. sp. *dianthi* race 2, was generated via transformation with a bacterial chitinase gene (ChiA) from *Serratia marcescens*. Unfortunately so far this success has not yet been translated to the field [39]. Genetic engineering is providing a valuable means of expanding the floriculture gene pool and promoting the generation of new commercial varieties. Extensive research has been conducted on the genetic transformation of different flowering plant species, and many ornamental species have now been successfully transformed, including those that are most important commercially. To date, more than 30 ornamental species have been transformed, including anthurium (*Anthurium cubensis*), begonia (*Begonia* spp.), carnation, chrysanthemum, cyclamen, datura (*Datura sanguinea*), gentian (*Genciana gorse*), gerbera (*Gerbera jamesonii*), gladiolus, hyacinth (*Hyacinthus orientalis*), iris (*Bulbous*), lilies, lisianthus (*Eustoma grandiflorum*), orchid (*Cattleya skinneri*), geranio (*Pelargonium domesticum*), petunia (*Petunia hybrida*), poinsettia (*Poinsettia* spp.), rose, snapdragon (*Antirrhinum majus*), and torenia (*Torenia* sp.) [40]. New ornamental plant varieties are being created by breeders in response to consumer demand for new products. In general terms, engineered traits are valuable to either the consumer or the producer. At present only consumer traits appear able to provide a return capable of supporting what is still a relatively expensive molecular breeding tool. Commercialization of genetically engineered flowers is currently confined to novel-colored carnations. The production of novel flower color has been the first success story in floriculture genetic engineering. However, further products are expected given the level of activity in the field. Other traits that have received attention include floral scent, floral and plant morphology, senescence of flowers both on the plant and postharvest, and disease resistance. To date, only a few ornamental genetically modified (GM) products are in development and only one, a carnation genetically modified for flower color, is in the marketplace. There are approximately 8 ha of transgenic carnation in production worldwide, largely in South America. The other breeding programs on color modification or alteration of plant architecture and height remain focused on rose, gerbera, and various pot plant species.

Selection of species and cultivars with natural genetic resistance to pathogens has been successful. Improving disease resistance has become an

important breeding objective in gerbera, one of the most important floricultural crops in the world. Development and application of molecular markers are expected to assist in the selection of gerberas with improved disease resistance [41]. Screening programs undertaken by several workers have reported resistance in many cultivars against *F. oxysporum* f. sp. *gladioli* causing yellows in gladioli [42–44]. With the help of cross-breeding, new gladiolus cultivars highly resistant to *Fusarium* were also developed [45–47]. One of the gladiolus cultivars designated as “Georgia Peach” is resistant to attack of *Fusarium* spp. and *Curvalaria* spp. “Georgia Peach” is also tolerant to the high temperatures experienced during Florida summer/early fall (August to October) and midwinter temperatures [48].

BIOCONTROL

Biological control involves the use of antagonistic microorganisms such as bacteria, yeast, and fungus. Botrytis blight caused by *Botrytis cinerea* is a difficult disease to control in many ornamental crops. The competitive saprophytes *Clonostachys rosea* and *Ulocladium atrum* have been evaluated with some success for suppression of *B. cinerea* on cyclamen [49], geranium [50], and rose [51–53]. In the presence of a high disease incidence (55%), the antifungal compounds as salts, oils, plant extracts, and the biocontrol agent *Ampelomyces quisqualis*, applied individually, provided satisfactory control of powdery mildew on roses. Most treatments were as efficient as the fungicide dodemorph [54]. Plant growth promoting rhizobacterial strain S2BC-2 (*Bacillus atrophaeus*) and a strain mixture, S2BC-2 + TEPF-Sungal (*Burkholderia cepacia*), inhibited the growth of *F. oxysporum* f. sp. *gladioli* (FOG). In comparison to the individual strain, the strain mixture showed maximum spike and corm production, with less vascular wilt and corm rot incidences in greenhouse and in field experiments [55]. Biocontrol with *C. rosea* applied to leaves and crop debris to reduce pathogen sporulation in a plastic greenhouse on two rose cultivars significantly reduced *B. cinerea* sporulation. However, disease incidence was not consistently reduced, probably because the applications on *C. rosea* started when Botrytis blight epidemic was advanced and no sanitation practices were performed on nontreated plots [56]. Microbial isolates from living petals, petal residues, and leaf residues of rose, and from laboratory collections, were evaluated for control of *B. cinerea*. Isolates of *Cladosporium oxysporum*, *Cladosporium cladosporioides*, and *Bacillus subtilis*, the most effective microorganisms against *B. cinerea* in flower buds, reduced the number of lesions in the range of 42–65%, compared with

59–89% for a standard fungicide (vinclozolin) [57]. The influence of application time and the length of exposure to sunlight on survival, establishment, and ability of *C. rosea* to suppress *B. cinerea* sporulation on rose debris were investigated in a climate-controlled greenhouse. Despite the effects of exposure to sunlight on *C. rosea*, the suppression of *B. cinerea* sporulation was only marginally affected, which shows the ability of *C. rosea* to withstand adverse environmental conditions and still provide suppression of *B. cinerea* sporulation on rose debris [58]. A pot experiment was conducted to study the interactions between the arbuscular mycorrhizal fungus (AMF) *Glomus fasciculatum* and the two pathogens *F. oxysporum* and *Colletotrichum gloeosporioides* and subsequent effect on growth, disease tolerance, and the changes in antioxidative ability in cyclamen plants under growth chamber condition. The results demonstrate that AMF has the ability to induce resistance against Fusarium wilt and anthracnose in cyclamen by increasing the antioxidative activity in plants, which promoted plant growth and biomass production and drastically reduced the disease incidence in cyclamen. [59]. Forty-five isolates of *F. oxysporum* f. sp. *dianthi* and another 45 of antagonistic fungi *Trichoderma* spp. and *Gliocladium* spp. were evaluated *in vitro*. Antagonistic fungi showed highly significant differences to invasion of the pathogen's colony, radial growth of the antagonist, and difference in radial growth *in vitro*. The cellulolytic activity *in vivo* of strains T25 and T47 was significantly higher in soil without disinfection; T47 also showed such activity in disinfected soil. Antagonistic fungi are an alternative to control “dormilona” of carnation caused by *F. oxysporum* f. sp. *dianthi*, to enhance biocontrol of pathogens, and to contribute to reduce the use of pesticides in the flower-producing zone [60]. Mishra et al. [61] reported integrated and biological control of gladiolus corm rot and wilt caused by *F. oxysporum* f. sp. *gladioli*. Isolate of *Trichoderma virens* and carboxin, individually and in combined form, significantly reduced disease incidence in both glasshouse and field conditions. Chandel and Tomar [62] biological control of gladiolus wilt caused by *F. oxysporum* f. sp. *gladioli* using different methods. The soil placement method proved effective compared to the corm dip method. *Trichoderma harzianum* in comparison to *T. viride* showed superior performance against wilt pathogen and resulted in minimum disease incidence in addition to improvement in growth parameters of gladiolus. An attempt to manage the disease with AM (Arbuscular mycorrhiza) fungi (*Glomus mosseae* and *Glomus etunicatum*) under pot culture conditions was made by Bhardwaj et al. [63]. In plants inoculated with *G. mosseae* experienced maximum disease reduction followed by *G. etunicatum* application. In this

study, significantly higher root length and root dry weight was recorded in mycorrhizal-inoculated plants than nonmycorrhizal plants. Biocontrol agents are likely to perform best when pathogen populations are low or when coupled with naturally suppressive potting mixes. Thus, growers who improve sanitation and cultural practices will see greater benefits from biocontrol agents and be able to reduce the amount of pesticide used in crop production.

CHEMICAL CONTROL

Fungicides continue to have an integral role in ornamentals disease management. Botrytis fungicides are generally first developed for use on grapes. Recent Botrytis control trials on poinsettia and geranium showed decreased disease incidence or severity in treatments with polyoxorim, fenhexamid, iprodione, and chlorothalonil sprays [64]. Currently, thiram is the only fungicide labeled for postharvest treatment of flower seeds in the United States. Thiram gave an 80% reduction in seed-borne anthracnose in the field crop of lupinus (*Lupinus albus*) [65]. Greenhouse populations of downy mildew, powdery mildew, and *B. cinerea* are particularly prone to develop resistance. Strains of *B. cinerea* resistant to benzimidazole fungicides or partially resistant to dicarboximides have been documented in North America [66–68]. This resistance has appeared in many ornamental crops worldwide. Some of the newer chemistries in use in the ornamentals industry are also vulnerable to the development of resistant Botrytis populations. Recently *B. cinerea* indicated resistance to anilinopyrimidines (pyrimethanil) [69,70]. For *Puccinia horiana*, agent of chrysanthemum white rust, resistance to fungicides in carboximide, triazole, and strobilurin classes has now been reported [71,72]. Resistance is also an important factor in the control of soil-borne diseases. A high incidence of mefenoxam resistance has been documented in greenhouse populations of *Pythium* [73] and *Phytophthora* [74,75]. The disease in gladiolus is controlled by treating corms with systemic fungicide before as well as after harvesting the corms. Wanial [76] reported that the combination of bavistin (0.1%) and difoltan (0.5%) gave the best control of wilt of gladiolus caused by *F. oxysporum* f. sp. *Gladioli*, better than the use of the fungicides individually. Gladiolus corms treated with Sportack (0.0125%) for 3 h before planting gave good control of Fusarium yellows [77]. The *Fusarium* pathogen usually remains in the vascular tissues near the core of the corms. Due to the presence of the suberin layer inside the corm, the fungicides usually cannot reach the core of the corms to kill the fungal

pathogens. As a consequence, the fungicides are only partially effective in killing the pathogen. The benzimidazole fungicides (benomyl, carbendazim, and thiophanates) have a high resistance potential against pathogens because they have a specific mode of action. The resistance is usually not associated with a significant loss of fitness of the pathogen. It occurs in populations of *B. cinerea*, *D. bryoniae*, *Fusarium*, and powdery mildews. Acute problems of resistance to dicarboximide fungicides (e.g., iprodione, procymidone, and vinclozolin) have arisen when fungicides are used intensively and exclusively over many seasons [78]. Isolates are moderately resistant and tend to be almost as fit as sensitive strains in the absence of fungicides. Restricting the number of dicarboximide treatments to no more than three crops in greenhouses where resistance is found is recommended, even in the absence of detectable resistant strains. When infection pressure is high, the usual recommendation is to alternately mix these fungicides with protectants such as chlorothalonil, captan, or with biocontrol, which do not usually select for resistance. Ergosterol biosynthesis inhibitors (EBIs) are a group of fungicides that include triazole, imidazole, and pyrimidine fungicides, and inhibit C14 demethylation and morpholines. Unlike the sharp, significant nature of resistance toward benzimidazoles and dicarboximides mentioned previously, the resistance toward EBIs develops in the form of slow shifts in the pathogen population. For instance, powdery mildews in greenhouses were controlled for several years by benzimidazoles, hydroxypyrimidines, pyrazophos, and EBIs. Resistance is known in populations of *Sphaerotheca fusca*, but the alternation of fungicides, which is practiced in many countries, is helping to deal with problem. It is generally recommended to rotate or mix EBI fungicides with fungicides from other groups as well as with biocontrol. The failure of disease control in greenhouses is exemplified by the history of gray mold epidemics. Multiple resistant isolates occur in greenhouses that bear the resistance toward benzimidazole, diethofencarb, dicarboximides, and ergosterol biosynthesis inhibitors [79,80]. The phenylamide fungicides that inhibit RNA synthesis were introduced in the late 1970s for Phycomycetes control. During the 1970s, *Psilocybe cubensis* was controlled mainly with protective applications of dithiocarbamates and chlorothalonil. In the early 1980s, the phenylamide metalaxyl was released and soon afterward resistant strains were selected. Metalaxyl-resistant strains seem to be more competitive than wild-type strains [81]. Resistance was found also in *Phytophthora infestans* on tomato and *Bremia lactucae* on lettuce. Antiresistance mixtures of metalaxyl with protectant fungicides were developed to cope with phenylamide resistance. In order to reduce the pressure toward development of

resistance in pathogen populations, it is usually better to limit the exposure of the pathogen to a group of fungicides. The extreme summer conditions do not interfere with the survival of fungicide-resistant isolates [82]. The number of applications of fungicides of the same mode of action has to be limited, especially against fungi with many cycles during the growing season. The present status of resistant pests or pathogens in greenhouses is often unknown; growers tend to apply excess amounts of chemical, and control is not achieved. The development of tools for monitoring resistance should facilitate the assessment of different management options. The risks and benefits of chemical application need to be weighed in each situation. In addition to the cost of chemical treatment, potential negative side effects of pesticide use, such as stress, resistance problems, and interference with biocontrol, must also be considered [83]. Certain fungicide treatments on healthy plants may have a negative effect on plant growth and possibly increase the length of time to bring the crop into flower [84].

ALTERNATIVES UNDER EXPERIMENTATION

Chitosan

The majority of the chitin (long-chain polymer of *N*-acetylglucosamine) produced for agricultural purposes is sourced from the exoskeletons of crustaceans farmed/harvested for human consumption, chiefly shrimp, crab, and lobster. In addition to possessing a high chitin content, the use of crustacean exoskeletons provides a way of utilizing a major source of waste in the shrimp farming industry. Accurate data on global crustacean farming do not exist, but the Food and Agriculture Organization of the United Nations estimate that in 2011 global crustacean production was 5.9 Mt [85], with 35–45% of this amount being discarded waste (head and thorax). This means that the global chitinous waste production from this source is 2.1–2.7 Mt, most of which could be productively utilized in agriculture chitosan typically derived from chitin (poly β -(1-4) *N*-acetyl-D-glucosamine), a major component of the exoskeleton of insects and crustaceans [86] is soluble in weak acid and once the alkali is neutralized, it can be safely applied to plants/soil as a solution or as a dry powder.

Effectiveness of Chitosan Against Fungal Pathogens

The antifungal activity of chitosan has been tested against several micro-organisms [87,88] and it has been shown to inhibit the growth of *Alternaria alternata* 50.6–70% under *in vitro* condition [89,90]. Chitosan has been

repeatedly found to exhibit potent antimicrobial activity [91,92], which has been attributed to its cationic properties and the disruption of potassium signaling in pathogens [93,94]. However, chitosan could also be acting by creating barrier films, chelating mineral nutrients and making them inaccessible to pathogens, and/or preventing the release of mycotoxins from the pathogen [95–97]. Although it is possible to show direct toxicity of pathogens in *in vitro* cultures, when chitosan is applied to field-grown crops it is less clear whether the effects observed are due to direct toxicity of chitosan to the pathogen, the induction of plant defenses, and/or the stimulation of beneficial microbes. Soil amendment with chitosan has repeatedly been shown to control fungal diseases in numerous crops, especially *Fusarium* wilts [98–100] and gray mold [101,102]. It is also of note that these studies show chitosan to be fungistatic against both biotrophic and necrotrophic pathogens. The control of oomycete pathogens has also been achieved with chitosan treatment, with *Phytophthora capsici* controlled on peppers, is despite oomycetes lacking chitinous cell walls, like true fungi (eumycota). In the study by Xu et al. [94] on *P. capsici* in peppers, it was reported that the main effect observed in the pathogen was the disruption of the endomembrane system, especially the integrity of the vacuoles. Chitosan has been approved as a biopesticide by the U.S. Environmental Protection Agency (EPA) as a food additive by the U.S. Food and Drug Administration (FDA). Chitin-based treatments show promise as alternatives to the use of synthetic pesticides on fresh produce in postharvest storage [103,104].

Effectiveness of chitosan applied curatively two times at 7-day intervals after the appearance of rose powdery mildew symptoms on most of plant parts, was about 32%. When compound was applied just after the first disease symptoms appearance, after 2-week protection its effectiveness was about 43–60%. After 4 weeks, effectiveness of chitosan (Biochikol 020 PC) increased and ranged from 67% to 77%. In the control of *Peronospora sparsa* on rose shrubs, growing in plastic tunnel, chitosan at conc. 0.025% was applied four times at weekly intervals. Effectiveness of chitosan against this pathogen was over 72%, similar to standard fungicide [105].

Chitin-based products have also been successfully used to extend the storage life of nonfood crops, including cut flowers [106]. Further advances in postharvest storage may also come from developments in chitin-based films and threads, which are being developed as biodegradable polymers and packaging [107]. The antimicrobial properties of the chitin in these products could further improve the shelf life of crops once they leave storage facilities [108].

PLANT GROWTH PROMOTION AND EFFECTS OF PLANT DEVELOPMENT

Improvements in plant growth that are thought to be independent of the effects on pest and disease control have been reported after the application of chitin-based treatment to a range of crops. Significant improvements in growth have been reported in ornamental crops, such as gerbera [109] and *Dendrobium* orchids [110]. In three studies on orchids it was found that chitosan was effective at a low concentration of 10 mg/L [110–112]. This indicates that chitosan was acting due to mechanisms other than simply improving nitrogen nutrition or as a carbohydrate energy source. Both Pornpeanpakdee et al. [111] and Nahar et al. [112] found that the growth of orchids (*Dendrobium* and *Cymbidium*, respectively) was enhanced when chitosan was supplied to micropropagated plants growing under aseptic conditions. This finding is backed up by other studies showing enhanced growth of the medicinal herb *Lippia dulcis* cultivated in liquid bioreactors [113].

In addition to increases in photosynthesis and vegetative growth, chitosan-based treatments have also been shown to modify developmental processes. Limpanavech and coworkers [114] studied the application of chitosan on *Dendrobium* orchids using a range of concentrations (1–100 mg/L) and a range of degree of deacetylation levels (70–90%) with all treatments inducing no changes in vegetative growth, but chitosan did induce precocious flowering. Utsunomiya and Kinai [115] also recorded precocious flowering and increased flower numbers when chitosan was applied to passion fruit (*Passiflora edulis*) as a soil drench. Induction of flowering by chitosan was again observed in *E. grandiflorum* [116], and in a separate study on *E. grandiflorum*, chitosan produced more intense petal pigmentation when dissolved into the holding solution of excised inflorescences [117]. Ramos-García and coworkers [118] evaluated the effect of the application of chitosan gladiolus corms, noting that the emergence of the corm, the number of flowers per spike, number of cormels, and shelf life were influenced positively. In orchid tissue culture, the growth of meristem explants into procorm-like bodies in liquid medium was accelerated up to 15 times in the presence of chitosan oligomer [119]. The effect of coating Freesia corms with natural polysaccharides such as chitosan and its derivatives on growth and plant health show that coating of corms clearly improves their health and reduces selected fungal infection [120]. The chitosan O-80 at concentration of 1 ppm and 100 ppm could induce early flowering and increase the accumulative inflorescence in *Dendrobium* “Eiskul”. The chloroplasts in the young leaves of the plants treated with chitosan were found to be

significantly larger than those of the non-chitosan-treated ones. The molecular investigation indicated that chloroplasts were one of the target sites for chitosan action in *Dendrobium* [121]. The effect of chitosan treatment on postharvest quality of cut flowers as *Lilium* Oriental hybrid “Siberia” and *Lilium* Asiatic hybrid “Dream Land” showed that vase life was increased. The sugar contents increased in petal, but decreased in leaf and ovary [122]. The effect chitosan treatment on growth and quality of cut *Dendrobium* “Sonia” showed that inflorescences sprayed with chitosan had the highest fresh weight and increased petal width [123]. Unfortunately, no information is available on the mechanism by which chitosan-based treatments induced flowering in these ornamental crops.

PLANT EXTRACTS AND ESSENTIAL OILS

Although, lacking an immune system comparable to animals, plants have developed mechanisms that help in defending themselves against infections [124]. In the case of fungal infection, these mechanisms include synthesis of bioactive organic compounds such as phenols, tannins, alkaloids, saponins, terpenoids, coumarins, glycosides, and steroids [125]. However, quantity and quality of these active compounds depend on the plant species, plant tissue, and environmental factors. Several works demonstrated in laboratory trials that natural products of higher plants may possess a new source of antimicrobial agents [126]. Several studies report the presence of several groups of compounds in various plant parts such as leaves, bark, fruit, root, and flowers. As of early 2013, more than 200,000 secondary metabolites have been identified from various living organisms (<http://www.chemnetbase.com/>). The total number of such compounds synthesized by all living organisms on earth is estimated to be much higher. Many of the identified secondary metabolites are of plant origin, and their number is estimated to be less than 10% of the total. In many cases, these compounds serve as plant defense mechanisms against predation by microorganisms, insects, and herbivores. Some, such as terpenoids, give plants their odors; other (quinones and tannins) are responsible for plant pigment. Many compounds are responsible for plant flavor (the terpenoid capsaicin from chili peppers) and some of the same herbs and spices used by humans to season food yield useful medicinal compounds.

Essential oils are aromatic oily liquids, obtained by hydrodistillation from whole tissues or seeds of plants and are usually mixtures of several components terpenes, sesquiterpenes aldehydes, ketones, and phenolic

components. The essential oils thymol, menthol, peppermint oil, citral, cinnamol, and various others are some of the phytochemicals that have been studied for antifungal activity. The essential oils reported in various studies exhibit antimicrobial, allelopathic, antioxidant, and bioregulatory properties [127,128]. The essential oils are obtained from different plants and have indicated fungicidal properties reported to suppress fungal growth and development *in vitro* and *in vivo* in different fresh produces [129–132], while also being safer for the environment than synthetic fungicides. Essential oils inhibit postharvest pathogens mainly due to their direct effect on the mycelial growth of the pathogens and spore germination by affecting the cellular metabolism of the pathogens [133–136]. The interactions of the essential oils with the cell membranes could result in leakage of some cellular components, such as the ATP, which is the main energy-storing molecule. Essential oils such as carvacrol, eugenol, and thymol, which contain more phenolic compounds in their chemical structure, exhibit the strongest antimicrobial properties against fungal pathogens [137]. Phenols can also interact with membrane proteins, which could result in deformations in the membrane structure and functions [138]. Mode of action of essential oils without phenolic groups such as lemongrass oil could be due to membrane disruption by the lipophilic compounds [139]. Deformation and dysfunction of the membrane could lead to interference in the generation of ATP within the fungal cell, mainly inhibiting the enzymes and substrate usage of ATP production [140,141]. Furthermore, the germination of spores and germ tube elongation could be affected and the mycelia growth of the fungi could be inhibited. Common observations regarding the alterations of mycelia or fungal spores include massive vacuolation of cytoplasm with vacuole fusion, the appearance of numerous lomasomes with folding, the detachment of the plasma membrane from the cell wall as well as the fibrillar layers of the cell wall becoming thinner, losing their integrity, and finally, failing to deposit on the cell wall [142]. The synergistic effect between the essential oils could be used to increase the antimicrobial properties by reducing their amount. During synergism, oxygenated compounds such as citral, thymol, and carvacrol are more active than *p*-Cymene (terpene hydrocarbons). However, the *p*-Cymene was reported to create swelling in the fungal cell membranes to facilitate the transportation of citral, thymol, or carvacrol into the cell, thereby demonstrating the synergistic effect of two different essential oil components being applied together [143]. Essential oils extracted from oregano (*Ocimum gratissimum*) were significantly more active against *Penicillium expansum* than those extracted from lemon grass

(*Cymbopogon citratus*) and thyme (*Thymus vulgaris*) [144]. Essential oils from *C. citratus*, *O. gratissimum*, and *T. vulgaris* were reported to possess many active compounds, including geranial, neral, thymol, terpinen-4-ol, linalool, and carvacrol [145]. The synergistic effects between these essential oils could be exploited in order to improve their antimicrobial activities and reduce their concentrations required to demonstrate a higher antimicrobial effect [144]. The presence of a variety of antimicrobial compounds in plant extracts and essential oils confers on them advantages such as having different modes of actions depending on the compound involved and thus hindering the development of resistance by the pathogen.

Control of Microorganisms with Plant Extracts and Essential Oils

Few studies regarding the use of plant extracts as a means of control of post-harvest diseases of ornamental plants have been reported. Antifungal activity of Aloe vera (*Aloe barbadensis miller*) leaves against *B. gladiolorum*, *F. oxysporum* f. sp. *gladioli*, *Heterosporium pruneti*, and *Penicillium gladioli* inhibited mycelial growth successfully [146]. Gomez and coworkers [147] reported the efficacy of antifungal activity of extracts of atalantia (*Fagara monophylla*) tree, its alkaloids components, and antibiotic properties were tested against *Aspergillus terreus*, *Aspergillus flavus*, *Penicillium digitatum*, *Penicillium funiculosum*, *Penicillium citrinum*, *Paecilomyces*, and *Candida albicans*. Pârveu and coworkers [148] showed antifungal activity of extracts of onion (*Allium senescens*) against *Aspergillus niger*, *B. cinerea*, *Baeonia paeoniae*, *F. oxysporum* f. sp. *Tulipae*, and *P. gladioli*. Antifungal activity of leaf extracts of wheat (*Triticum aestivum*), corn (*Zea mays*), sunflower (*Helianthus annuus*), pepper (*Capsicum annum*), onion (*Allium cepa*), and marigold (*Tagetes erecta*) was assessed by Riaz and coworkers [149] against *F. oxysporum* f. sp. *gladioli*. Marigold extract and sunflower were effective at all concentrations used to significantly reduce fungal biomass in 54–79%, 33–85%, and 45–57%, respectively, concluding that the aqueous extracts of these three species contain antifungal constituents to control this fungus. In the case of onion extract, only the highest concentration suppressed the fungal biomass by 73%.

Garduño-Pizaña and coworkers [150] evaluated the *in vitro* fungicidal or fungistatic activity of powders and aqueous, methanolic, hexanic extracts (5%) of 15 Mexican plants species on the development of *F. oxysporum* f. sp. *gladioli*. The hexane extract epazote (*Chenopodium ambrosioides*), the methanol extract of Mexican plum (*Spondias purpurea*) and guava (*Psidium guajava*), and the aqueous extract of gourd (*Leucaena esculenta*) and cuahulote

(*Pithecellobium dulce*) inhibited the mycelial growth in more than 50%. In addition, powders of nanche (*Byrsonima crassifolia*) decreased the germination percentage and sporulation of the pathogen. All species showed antifungal activity as methanol extract. The conclusion was that the high chemical diversity of plant species analyzed differently affected fungal growth, possibly for the individual compounds or synergism of them. Chohan and coworkers [151] evaluated the *in vitro* fungitoxic effect of *Azadirachta indica*, *Ocimum basilicum*, *Datura stramonium*, *T. erecta* and *Allium sativum* on the growth of *F. oxysporum* f. sp. *gladioli*. *A. indica* extract was effective in all concentrations used, and mycelial growth was inhibited in 83.5, 57.5, 46, and 34.5% at 8% concentration. *T. erecta* and *D. stramonium* also at 8% concentration showed a mycelial growth inhibition of 58.5 and 35%, respectively.

Dwivedi and Neetu [152] evaluated the antifungal activity of the extract of clove (*Eugenia caryophyllata*), marango (*Moringa oleifera*), ajwain (*Trachyspermum captivum*), and ginger (*Zingiber officinale*) against *Fusarium solani* at concentrations of 10, 25, and 50%. *E. caryophyllata* extract completely inhibited the growth of *F. solani* mycelia in the third, fifth, and seventh day at a concentration of 50% compared to control. As concluded in this study, the extract of *E. caryophyllata* presented mycelial growth inhibition according to concentration. The powders and leaf extract of five Mexican plants at 5% (hexane and methanol) revealed remarkable antifungal effect *in vitro* on the growth inhibition of *F. oxysporum* f. sp. *gladioli* with a range of 15–67%. Leaf powders and extracts contained sesquiterpenes (46.0%), fatty acids (23.6%), diterpenes (14.6%), phenolic compound (11.23%), and monoterpenes (4.7%). The high chemical diversity of the analyzed plant species results in different effects on the development of the fungus [153]. The bulb and stem rot of hybrid lily (*Lilium* spp.) is characterized by necrosis upwardly; it causes the premature death of plants, as well as 50% loss in production. The causal agent was *F. oxysporum*. The biological efficacy of three chemical and biological fungicides in greenhouse was evaluated, observing that the best products to control the fungus were prochloraz (SPORTAK®) and *T. harzianum* with 89 and 82% efficiency, respectively [154]. Twenty-four extracts (aqueous and ethanolic) from nine plant species were evaluated against *Alternaria chrysanthemi*, the causal agent of leaf spot of chrysanthemum. The highest antifungal activity was shown by ethanolic and aqueous extracts of kahum (*Acalypha gaumeri*) root [155]. The antifungal activity of gobernadora (*Larrea tridentata*) hidrosoluble resin extract and chitosan solutions alone and in combination demonstrated their fungicidal effect against *B. cinerea*, *Colletotrichum coccodes*, and *F. oxysporum* f. sp. *lycopersici* [156].

The essential oils produced by plants have been reported to inhibit post-harvest fungi under *in vitro* conditions. Barrera and Garcia [157] evaluated nine essential oils and their compounds in bioassays of inhibition of mycelial growth of *Fusarium* sp. The oil of thyme (*T. vulgaris*) had the best antifungal effect by showing complete inhibition of mycelial growth at doses of 200, 250 and 300 $\mu\text{g/mL}$. The oils of cinnamon (*Cinnamomum zeylanicum*), clove (*Syzygium aromaticum*), and epazote (*Teloxys ambrosioides*) decreased the rate of mycelial growth by increasing the dose to 100–300 mg/mL . Moreover, the carvacrol compounds, thymol and cinnamaldehyde, showed the best inhibition of mycelial growth at all concentrations tested. Furthermore, Sharma and Tripathi [158] tested the effectiveness of oil chan (*Hyptis suaveolens*) on growth and morphogenesis of the fungus *F. oxysporum* f. sp. *gladioli* to completely inhibit the mycelial growth of the fungus (0.9 and 0.7 mg/mL) and germination of conidia (0.4 mg/mL). The differences observed by a study of light microscopy (400 $\times$) were decreased and deformation of conidia, loss of pigmentation, and abnormal hyphae such as thinning, granulation, and retraction of cytoplasm. The application of essential oils has been studied to inhibit mycelial growth of *F. oxysporum* f. sp. *gladioli*, obtaining good results *in vitro* with *C. zeylanicum* oils, *T. vulgaris*, and *S. aromaticum* at 100, 150, 200, 250, and 300 ppm. [159]. The vase-life of gerbera cv. “Dune” (*G. jamesonii*) flowers held in a solution containing silver nanoparticles (SNP) and essential oils of carvacrol plus 6% sucrose was found to be significantly higher than control treatments, which suggests the potential application of essential oils or SNP as novel alternatives to common chemicals used in preservative solutions for gerbera flowers [160].

Antifungal assays showed that the seed oil of *Jatropha curcas* at 2.5 mg/mL inhibited the growth rate of *F. oxysporum* f. sp. *gladioli* to about 0.77 cm/day , while the effect of the G2–G9 fractions, obtained by column chromatography, tested on this parameter was variable. The best inhibitory concentration for G3–G9 fractions was 200 mg/L . The major fatty acid was oleic acid (49.0%) and was the major component in G2–G9 fractions. The lowest percentage germination was with the commercial triolein at 500 mg/L (30%) [161]. Vapors of thyme, oregano, and lemongrass and their major components showed complete growth inhibition of *B. cinerea* and *Alternaria arborescens*, while *Geotrichum candidum* was more sensitive to citral. Emulsions of oils of thyme and oregano as dip treatments reduced disease development in tomatoes inoculated with *B. cinerea* and *A. arborescens* [162]. In experiments *in situ* the soil application of allelopathic plants leaves was used in an *in vivo* experiment by Riaz and coworkers [163] for handling corm rot. Plants

successfully used were eucalyptus (*Eucalyptus citriodora*), gramilla (*Coronopus didymus*), lambsquarters (*Chenopodium album*), and nut grass (*Cyperus rotundus*). For the particular case of *Penicillium*, different methods have been tested with the application of secondary metabolites in tulip (*Tulipa praestans*) bulbs. Smid and coworkers [164] made 3.9 mM solutions cinnamaldehyde, reducing disease incidence by 50% during the time of storage. In greenhouse experiments, healthy corms were inoculated with spore solution of *F. oxysporum* f. sp. *gladioli* and treated with the methanolic extracts of purple plum (*S. purpurea*), guava (*P. guajava*), and nanche (*B. crassifolia*) at 2.5 and 5%. The extract of *S. purpurea* at 2.5% showed the highest emergency days and highest severity of disease. The extract of *P. guajava* at 5% developed flower spike in 75% of plants compared with fungicide control (33%). The treatment of *B. crassifolia* at 2.5% showed the lowest emergency days and higher height of the plant compared to the other treatments [165]. *Penicillium* causing storage gladiolus corm rot was controlled as alternative management, with hexane extracts of epazote and methanol extracts of guava. The methanol extract of guava at 5% showed damage reduction as curative or preventive treatment in storage conditions, the corm's viability was not affected [166]. In a field experiment, two varieties of gladiolus corms treated with methanol extract of *P. guajava* at 5% emerged in fewer days, showed highest height, accelerated flowering, increased the number of flower buds, and had higher storage life at 11 and 13°C. While the treatment with chitosan showed lower incidence and severity in plants and higher storage life at 11°C [167]. Storage of tulip bulbs in atmospheres containing cinnamaldehyde, perillaldehyde, salicylaldehyde, or carvone resulted in a significant reduction of the natural *Penicillium hirsutum* infection. Dipping tulip bulbs in an aqueous solution of 3.9 mM cinnamaldehyde gave a 40-fold reduction of the fungal population but did not affect the bacterial population and had no effect on the total stalk length or the flowering capacity of tulip bulbs [168]. In field conditions, gladiolus rust caused by *Uromyces transversalis* was treated with ethanol extracts of cauliflower, broccoli, and radishes at the concentration of 0.1% and had a lower percentage infection [169].

CONCLUDING REMARKS

Chemical control has been a routine practice in management of diseases pre- and postharvest in ornamental crops. But application of chemicals is associated with many side effects such as the development of resistance to pathogen, new races of pathogen, depleting of soil fertility, hampering

beneficial microbiota, and interference with biocontrol. Certain fungicide treatments on healthy plants may have a negative effect on plant growth and possibly increase the length of time to bring the crop into flower and negative effects on the environment as a whole. Searching for alternatives in the disease management of ornamental plants would be an appropriate approach. Use of resistant cultivars, biocontrol agents, chitosan, plant extract, and essential oils alone or in combined forms can enhance the efficiency of management strategies against diseases of ornamental crops. Substantial progress has been accomplished in evaluating new physical, low-toxicity chemical and biological alternative control methods as part of management of diseases programs. GRAS (generally recognized as safe) compounds, natural compounds (essential oils, plant extracts, chitosan), biocontrol agents and resistant cultivars are nonpolluting alternative control treatments that have shown potential value, ultimately improving crop yield and quality. The effectiveness of chitosan-based treatments has been found to be comparable to those achieved with current synthetic pesticides and fertilizers. This effectiveness combined with the low cost, low concentration required, ample supply (recycled waste), and health/environmental safety lead to a forecast that a range of chitin-based/augmented products will become a more common feature in agriculture in the near future. Research work to find new and more effective alternatives and especially to integrate different treatments in a multifaceted approach is in progress, as advances in the knowledge on the molecular and biochemical mechanisms underlying host-pathogen interaction and how they are influenced by potential post-harvest treatments are achieved, more tools will be available for the ornamental industry to design tailored cost-effective management of diseases pre- and postharvest for the control of pathogens.

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CHAPTER 9

Morphological and Ultrastructural Modifications of Chitosan-Treated Fungal Phytopathogens

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INTRODUCTION

Fungal parasites are by far the most prevalent plant pathogenic organism. All horticultural commodities are attacked by a low or high number of phytopathogenic fungi. They can infect many different kinds of plants at any point in their preharvest stage, and also infect fruit and vegetables through their postharvest life. In most instances, they may be controlled through the use of fungicides; however, there are various disadvantages about their application, including the following: (i) their increasing ineffectiveness against resistant fungal strains, (ii) soil and underground water contamination, and (iii) harmful effects to nontarget organisms (e.g., domestic and wild animals and beneficial insects) [3,4,59]. In addition, fungicides today are under strict scrutiny, and their use is heavily regulated [1,6]. These control measures have spurred the effort to develop other control alternatives, which include the application of natural products such as chitosan [11,29]. In line with this concept, a number of studies have documented that chitosan, the deacetylated form of chitin, extracted from diverse marine organisms, insects, and some fungi can exert significant fungicidal effects on different phytopathogenic fungi that affect a wide array of horticultural commodities [7–10] (Table 9.1) through inducing serious morphological and ultrastructural damages to the hyphae and conidia of pathogenic fungi, eliciting a number of structural defense reactions by the infected plants. In this review, a general definition of a fungus and the cellular structures and function of a fungal cell are provided. The mechanisms by which chitosan is thought to directly act on microorganisms are also highlighted. Then a number of findings related to the effect of chitosan on the fungal morphology and cell ultrastructure of the pathogen alone and during the plant-host interaction through the use of microscopic

Table 9.1 Summary of *in vitro* effect of chitosan on various phytopathogenic-treated fungi and level of control

Fungi	Chitosan concentration	Degree of control	References
<i>A. alternata</i>	0.5, 1.0, 1.5, 2.0, 2.5% w/v chitosan low, medium, and high molecular weight	Inhibition by 50% only at 2.5% and no sporulation at this concentration with chitosan medium molecular weight	[68]
<i>Aspergillus flavus</i>	0.0, 1.0, 2.0, 3.0, 4.0, 5.0 g/L	Best concentration at 3.0 and 3.5 g/L = 73% inhibition of growth and 50% spore germination	[60]
<i>A. niger</i>	Variation in molecular weights (50, 140, 200, 800, 1,000 kDA)	Antifungal rate: 50 = 98.2%, 140 = 82.3%, 200 = 73.5%, 800, 1,000 = growth increased	[53]
<i>B. oryzae</i>	Chitosan INCA and SIGMA (0, 300, 500, 700, 1000 mg/L)	100% mycelial inhibition with commercial chitosan sigma at 1,000 mg/L	[67]
<i>B. cinerea</i>	0.0, 0.01, 0.1, 1%	–No effect on conidial germination –Growth rate of 15–30 mm/day, depending on concentration	[55]
<i>C. destructans</i>	0.5, 1.0, 2.0 mg/mL	Radial growth inhibition according to concentration: 0.5 = 23%, 1.0 = 43%, 2.0 = 71%	[49]
<i>C. floridanum</i>	0.5, 1.0, 2.0 mg/mL	Radial growth inhibition according to concentration: 0.5 = 20%, 1.0 = 41%, 2.0 = 56%	[49]
<i>Cladosporium cucumerinum</i>	50, 500, 1000 µg/mL	Mycelial inhibition index: 50 and 1,000 µg/mL = 20%, 500 µg/mL = 30%	[66]
<i>C. gloeosporioides</i>	0, 0.5, 2.0, 2.5%	Conidial germination the most affected parameter No germination at 2.0% concentration	[5,7,8,54]
<i>Colletotrichum musae</i>	0.10, 0.20, 0.30, 0.40, 0.50, 0.75, 1.0, 1.5, 2.0% (w/v)	Total mycelial growth inhibition at > 0.75% concentration	[44]
<i>F. acuminatum</i>	0.5, 1.0, 2.0 mg/mL	Radial growth inhibition according to concentration: 0.5 = 7%, 1.0 = 21%, 2.0 = 27%	[49]

<i>F. oxysporum</i>	0.30, 0.75, 1.5, 3.0, 4.5 g/mL	Radial growth inhibition according to concentration: 100% at 4.5 g/mL	[64]
<i>F. oxysporum</i> f. sp. <i>radicis-lycopersici</i>	0.5, 1.0, 2.0 mg/mL	Growth rate according to concentration: 0.5 = 61.4%, 1.0 = 61.9%, 2.0 = 22.8%	[56]
<i>F. solani</i>	0.05, 0.10, 0.2%	Inhibition of sporulation, germination, and length of germ tube increased with concentration	[14]
<i>Fusarium verticillioides</i>	Low, medium, and high viscosity (1.0, 2.0, 3.0, 4.0 g/L)	Highest inhibition with chitosan low viscosity. 30 g/L = 50% (CQ ₅₀)	[62]
<i>Gaeumannomyces graminis</i> var. <i>trici</i>	0.5, 1.0, 2.0 mg/mL	Growth rate according to concentration: 0.5, 1.0 = 102.6%, 2.0 = 92.7%	[56]
<i>Leptographium procerum</i>	Low molecular weight and oligochitosan both at 0.05 and 0.075%	Higher control with oligochitosan at 0.05% = 90%; 0.075% = 100% mycelial inhibition	[23]
<i>Macrophomina phaseolinae</i>	Low and high molecular weight at 1, 2, 3, 4, 5 g/L	Low molecular weight at 5 g/L = 83.3%. High molecular weight > 4 g/L = 100% inhibition	[37]
<i>Monilinia fructicola</i>	50, 500, 1,000 µg/mL	Mycelial inhibition index: 50 µg/mL = 20%, 500 µg/mL = 25%, 1000 µg/mL = 50%	[66]
<i>P. digitatum</i>	0, 0.5, 1.5, 2.5, 3.0%	100% mycelial inhibition at 2.5 and 3.0% concentrations	[7]
<i>Pythium ultimum</i>	0.5, 1.0, 2.0 mg/mL	Growth rate according to concentration: 0.5 = 99.5%, 1.0 = 63.4%, 2.0 = 23.7%	[56]
<i>P. botryose</i>	0.1, 0.2, 0.5, 1.0, 2.0 mg/mL	Inhibition rate: 0.1 = 47.7%, 0.2 = 60.9%, 0.5 = 87.5%, 1.0 = 90.5%, 2.0 = 91.2%	[74]
<i>Pyricularia grisea</i>	Low molecular weight chitosan, O-acyl chitosan derivatives 0–6 g/L	100% inhibition at 5 g/L	[12]
<i>Rhizoctonia solani</i>	Low and high molecular weight at 1, 2, 3, 4, 5 g/L	Low molecular weight at 5 g/L = 100%. Low molecular weight > 3 g/L = 100%	[37]

(Continued)

Table 9.1 Summary of *in vitro* effect of chitosan on various phytopathogenic-treated fungi and level of control (*cont.*)

Fungi	Chitosan concentration	Degree of control	References
<i>R. stolonifer</i>	0, 3, 5 g/L	Low effect at any concentration	[79]
<i>S. sclerotiorum</i>	Low and high molecular weight at 1, 2, 3, 4, 5 g/L	Low and high molecular weight at 5 g/L = 88.8% and 100% inhibition, respectively	[37]
<i>Sphacelotheca reiliana</i>	Commercial chitosan. Biochikol 020 PC: 1, 10, 100, 1000, 10,000 µg/L	Inhibition of mycelial growth, sporidia, and teliospores germination, according to concentration	[48]
<i>Sphaceloma apelinum</i>	Three chitosans: 1,000, 10,000, 100,000 ppm	No colony formation at 100,000 ppm	[72]
<i>S. sapinea</i>	Low molecular weight and oligochitosan both at 0.05%, 0.075%	Significant inhibition of germination with oligochitosan at both concentrations	[23]
<i>Ustilago maydis</i>	Commercial chitosan Biochikol 020 PC: 1, 10, 100, 1000, 10,000 µg/L	Inhibition of mycelial growth, sporidia, and teliospores germination, according to concentration	[48]
<i>Verticillium dahliae</i>	0.5, 1.0, 2.0 mg/mL	Growth rate according to concentration: 0.5 = 131.2%, 1.0 = 113.8%, 2.0 = 45.7%	[56]

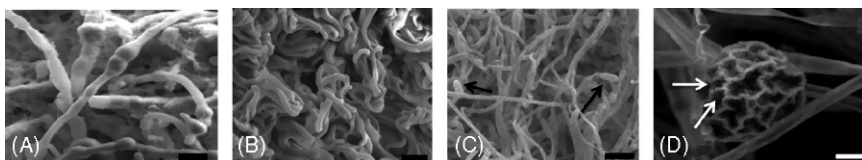


Figure 9.1 Fungal Hyphae Forming Mycelia. (A) *A. alternata* and (B) *Bypolaris oryzae*. Conidia and mycelia of (C) *F. oxysporum* f. sp. *gladioli*, and (D) sporangium and sporangiospores of *R. stolonifer*.

instruments, including optical and scanning and electron microscopy, are reviewed. Finally, this chapter concludes by outlining the most crucial effects of this polymer on the fungal cell and during the plant–pathogen interaction. Recommendations for further research in this area are also included.

FUNGUS DEFINITION

Fungi are small, usually microscopic organisms composed of living filaments called *hyphae*, growing in branches that form a mass or mycelia whose size and shape is variable according to genus and species (Figure 9.1A–C). They are small eukaryotic organism (cells contain a nucleus and organelles enclosed within a membrane), bearing spores without of chlorophyll (Figure 9.1C–D). Fungi secrete extracellular enzymes that degrade organic compounds and absorb nutrients through their cell walls, and they require an outside food source because they are not able to produce their own food [1,70].

FUNGAL STRUCTURES AND FUNCTIONS

As a eukaryotic organism, most fungi have complex cellular organization. From the outside in, the fungal cell is composed of an outermost layer called a *cell wall* (Figure 9.2A); this organelle allows the fungal cell to interact with its environment, protecting the cell from changes in osmotic pressure and environmental stresses and providing mechanical strength [19,38]. The underlying *plasma membrane* (Figure 9.2B) maintains the water, gas, and ionic balance between the cell interior and the environment through passive and active transport processes [75]. The *cytoplasm*, another structure of the fungus is composed of the cytosol, a liquid material where fungal organelles are suspended, and whose main functions are associated with protection from external damage and transport of genetic material and the products of cellular respiration (Figure 9.2C). Other specialized parts of the cell are the *organelles*, each having its own function [21,25,28]. However, it is reported that their major functions are involved in cellular

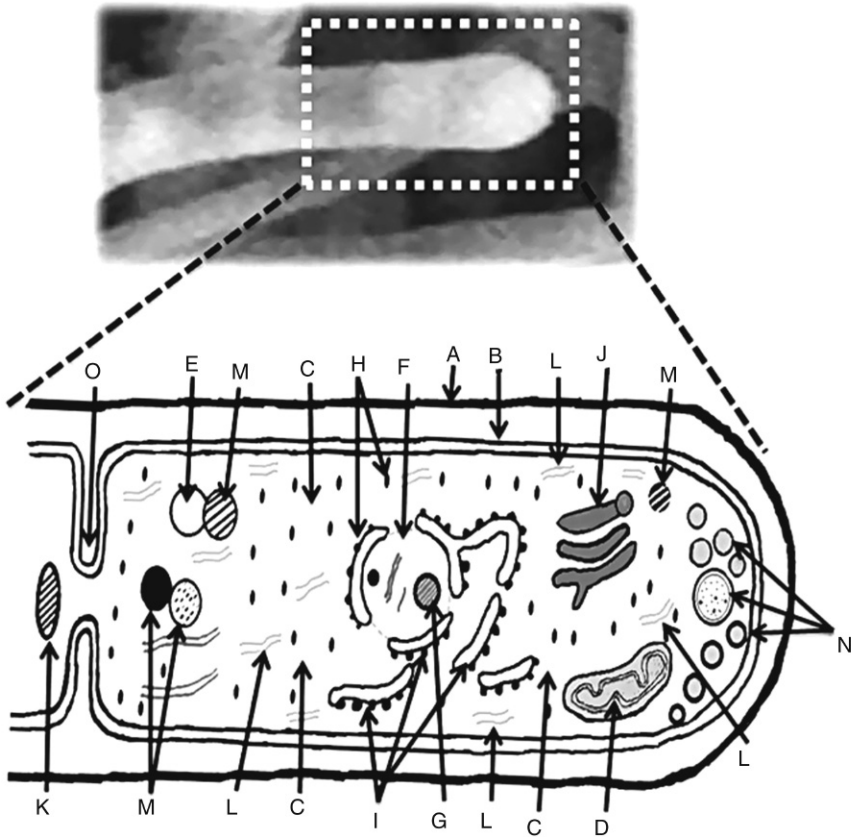


Figure 9.2 Typical Fungal Structure. (A) cell wall, (B) plasma membrane, (C) cytoplasm, (D) mitochondrion, (E) vacuoles, (F) nucleus, (G) nucleolus, (H) ribosomes, (I) endoplasmic reticulum, (J) Golgi apparatus, (K) Woronin body, (L) microtubule network, (M) specialized vesicles, (N) apical vesicles, and (O) septum.

respiration, the creation of new proteins and destruction of waste material. Among the most important organelles of the fungal cell are the *mitochondria*, considered the site of cellular aerobic respiration and antioxidative phosphorylation (Figure 9.2D); the *vacuoles*, whose main function is to store and recycle materials and regulate fungal cell pH (Figure 9.2E); the *nucleus*, which contains the cell's genetic material and maintains the integrity of fungal genes and regulates gene expression (Figure 9.2F); and the *nucleolus*, which stores RNA and makes products for manufacturing ribosomes (Figure 9.2G). Other important organelles of the fungal cell are the *ribosomes*, whose structures manufacture proteins in biological cells using RNA and amino acids (Figure 9.2H); the *endoplasmic reticulum*, which acts

as an intracellular transport of materials such as proteins around the cell (Figure 9.2I); and the *Golgi apparatus*, which receives proteins and other newly formed materials from the endoplasmic reticulum and packages and distributes them to other parts of the cell. This organelle produces a great quantity of vesicles (Figure 9.1J); the *Woronin body*, which carries out cell wall repair activities, blocking the septal pores after rupturing the fungal cell wall (Figure 9.2K), and the *microtubule network* associated with transport processes along the hyphae (Figure 9.2L). Specialized vesicles also found in the fungal cell are the *glyoxysomes*, which contain oxidative enzymes associated with diverse metabolic pathways, the *lysosomes*, which act as a biological catalyst and break down complex molecules (it is considered the main digestion site) (Figure 9.2M), and a body termed the “*Spitzenkörper*,” which consists of a cluster of small membrane-bound apical vesicles embedded in a meshwork of actin microfilaments that participate in cell wall biosynthesis and fungal morphogenesis (Figure 9.2N). The cross-wall structure that divides the fungal mycelium into chambers is named the *septum* (Figure 9.2O) [13].

PROPOSED MECHANISMS OF ACTION OF CHITOSAN ON FUNGI

The antimicrobial activity of chitosan on pathogenic microorganisms depends on different factors including, among others, the molecular weight, concentration, degree of deacetylation, and type of chitosan [10,47,63]. Today there is clear evidence about the mechanisms by which chitosan acts on fungi (Figure 9.3). As indicated by numerous studies, changes in fungal cell permeability are due to the interaction between the polycationic nature of the chitosan amino group and the electronegative charges in the outer surface of the fungal membrane, with this electrostatic interaction depending on the composition of the plasma membrane, having higher affinity in sensitive fungal membranes containing a polyunsaturated fatty acid composition [30,57,58]. In other studies, Peña et al. [61] concluded that the “strong binding” of chitosan to the membrane of the microorganisms leads to serious cellular imbalances of ion homeostasis K^+ and Ca^{2+} , causing the efflux of small molecules, including phosphates, nucleotides, and substrate of enzyme reactions that eventually affect fungal respiration and fermentation. Another theory suggests that chitosan interferes with the synthesis of mRNA and proteins through its penetration into the fungal nuclei [40,41,73] and acts as a chelating agent of metals and essential nutrients, inducing growth inhibition and starvation of fungi [24,65].

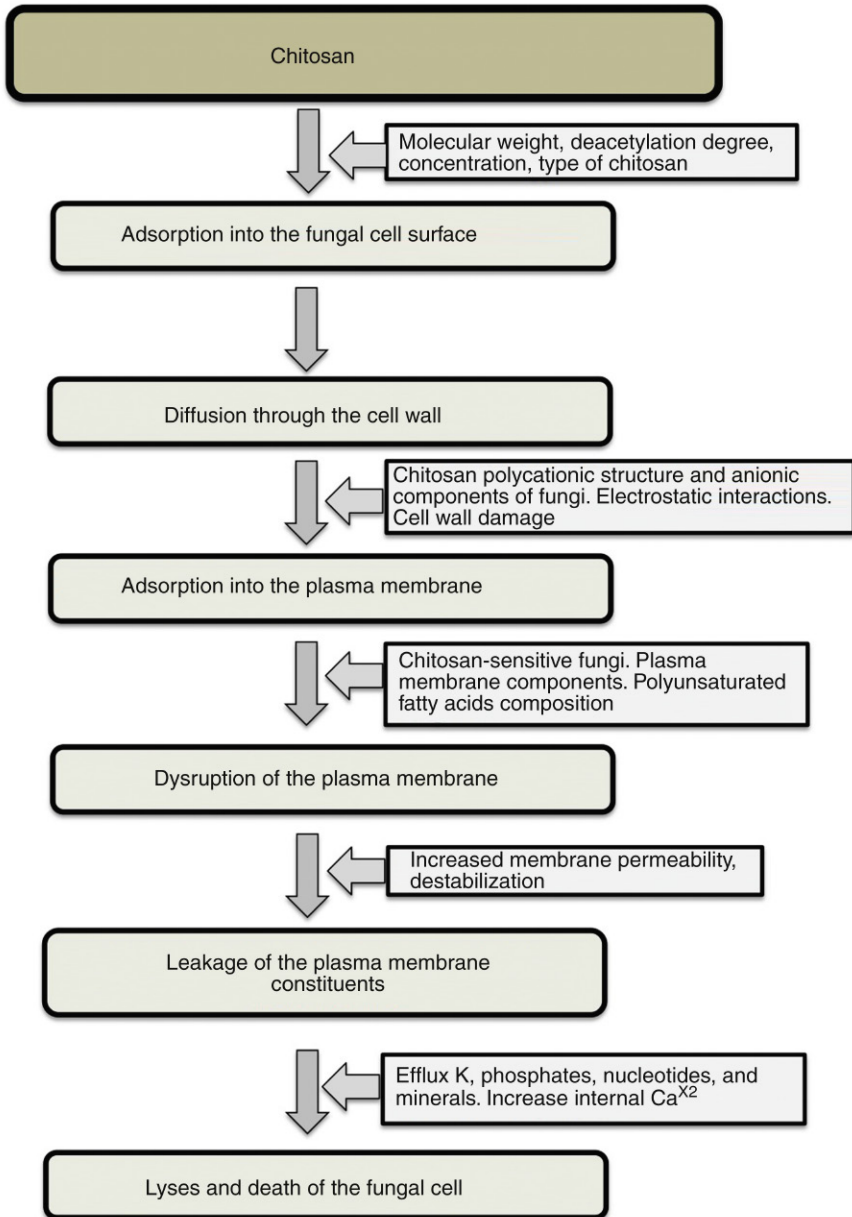


Figure 9.3 *Modes of Action of Chitosan over the Target Fungi.*

IN VITRO MORPHOLOGICAL ALTERATIONS INDUCED BY CHITOSAN ON FUNGI

As reported in the literature review, overall, chitosan induces severe morphological changes. Light microscopy examinations of chitosan-treated fungi have demonstrated that this compound at concentrations ranging from 0.5 mg/mL to 3 mg/mL exerts significant alterations on the hyphal surface of the treated fungi (Table 9.2). Observation of hyphae in fungi treated with chitosan, including *Botrytis cinerea*, *Sclerotinia sclerotiorum*, and *Rhizopus stolonifer* [22,31,32], showed, among other alterations, an excessive branching of the hyphae. Other significant changes reported in hyphae of various species of the genus *Fusarium* were the presence of vesicles and an increase in vacuolation [36,49]. The total leakage of cytoplasm by intense activity of chitosan on the fungi *B. cinerea*, *Cylindrocladium floridanum*, *Cylindrocarpon destructans*, *Fusarium acuminatum*, *F. oxysporum*, and *S. sclerotiorum* was also reported by Laflame et al. [49], Chea et al. [22], El Hassni et al. [36], and Ait Barka et al. [2].

Scanning electron microscopy (SEM) examinations have also provided evidence of the changes that take place in several fungi after exposure to different chitosan concentrations (Figure 9.4A–D) (Table 9.3). In general, SEM micrographies showed intense variations in the surface morphology of the treated fungi. Observations of the hyphal surface of numerous postharvest fungi such as *Alternaria alternata*, *Colletotrichum gloeosporioides*, *Penicillium expansum*, *R. stolonifer*, and *B. cinerea*, among others, grown on nutrient media amended with chitosan at concentrations ranging from 0.05% to 1.5%, have shown dramatic damage including surface distortion, corrugation, abnormal shapes, deformation, swelling and convoluted hyphae [11,26,27,65,68]. Intense dehydration of this fungal organ was also observed in *A. alternata*, f. sp. *gladioli*, and *R. stolonifer* at chitosan concentrations of 1.0 mg/mL [11,65,68]. For fungal conidia of *Aspergillus niger* and *R. stolonifer*, and oogonia of *Phytophthora botryose*, chitosan-treated SEM examinations also showed aggregations, abnormal shapes, and size reduction, respectively [45,60,74].

IN VITRO STRUCTURAL ALTERATIONS INDUCED BY CHITOSAN ON FUNGI

Cellular alterations of chitosan-treated phytopathogenic fungi have been further complemented by transmission electron microscopy (TEM) observations. Through these examinations at high magnification, it has been

Table 9.2 Summary of *in vitro* morphological alterations of chitosan-treated phytopathogenic fungi observed by optical microscope technique

Fungi	Chitosan concentration	Fungal structure	Morphological alterations	References
<i>B. cinerea</i>	> 1.5 mg/mL	Hyphae	Excessive branching	[32]
<i>B. cinerea</i>	Chitogel 1.75%	Hyphae	Cells devoid of cytoplasm, vesicle formation	[2]
<i>C. floricolum, C. destructans, F. acuminatum, F. oxysporum</i>	0.5, 1.0, 2.0 mg/mL	Hyphae	Cytoplasm aggregations, dissolution of protoplasm	[49]
<i>F. oxysporum</i> f. sp. <i>albedinis</i>	1 mg/mL	Hyphae	Presence of vesicles, excessive vacuolation, and empty cells	[36]
<i>F. oxysporum</i> f. sp. <i>radicis-lycopersici</i>	1, 3 mg/mL	Hyphae and conidia	Abnormal shape and contorted hyphae; no changes in conidia	[15]
<i>R. stolonifer</i>	1.5 mg/mL	Hyphae	Excessive branching	[32]
<i>S. sclerotiorum</i>	1, 2, 4% (w/v)	Hyphae	Excessive branching, abnormal shape, and empty cells	[22]

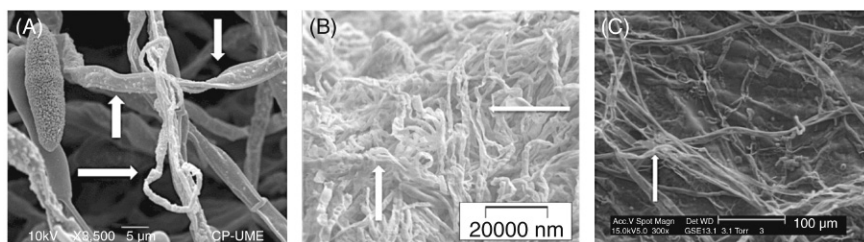


Figure 9.4 Scanning Electron Micrographies. (A) *A. alternata*, (B) *F. oxysporum* f. sp. *gladioli*, and (C) *R. stolonifer* treated with chitosan at 1.0%, showing distorted, thinner, and irregular shape of mycelia.

possible to observe serious structural damages in hyphae and conidia when exposed to chitosan (Figure 9.5A–D and Figure 9.6A–D). In general, the degree of cellular damage correlated with the chitosan concentration applied (Table 9.4). From the outside in, the observed changes varied from loosened, broken, and uneven cell walls to a serious “exfoliation” of this organ, as observed on *A. alternata*, *A. niger*, *R. stolonifer*, and *Sphaeropsis sapinea* [31,52,65,69,77]. The retraction and disintegration of the plasma membrane and increased and extended vacuolization throughout the fungal cell were consistent modifications reported for most treated fungi, but in some cases as those reported for *C. destructans* [49] and *Phytophthora capsici* [78] examinations showed, size increase of vacuoles, thickening of plasmalemma, and appearance of tubular material. In addition, in some fungal cells, including, among others, *A. alternata*, *Trichoderma harzianum*, and *F. oxysporum* f. sp. *radicis-lycopersici* [15,52,56,71], the cytoplasm appeared disorganized with loss of integrity and in some cases aggregations of fibrillar and electron dense material. Examinations of fungal cells subjected to chitosan concentrations of 1.5 mg/mL showed hyphae severely damaged with leakage of cytoplasm, no visible organelles, and lysis of the cell [15,49,69].

CYTOLOGICAL CHANGES INDUCED BY CHITOSAN IN DIFFERENT FUNGAL PATHOSYSTEMS

The interaction between fungi and their chitosan-treated hosts has been examined under optical and TEM microscopies. As shown in Table 9.5, chitosan causes severe morphological damage to the pathogenic microorganism, halting in most cases its development to external tissues: as epidermis cells and outer cortex of the host. Ultrathin sections of the interaction between *B. cinerea* and bell pepper fruit treated with chitosan have shown minor modifications of the host, with appearance of external fibrillar dense

Table 9.3 Summary of *in vitro* morphological alterations of chitosan-treated phytopathogenic fungi observed by SEM microscope technique

Fungi	Chitosan concentration	Fungal structure	Morphological alterations	References
<i>A. alternata</i>	2.5% medium molecular weight	Hyphae	Distortion, swelling, and nodulation	[69]
<i>A. alternata</i>	500 µg/mL	Hyphae	Swelling, abnormal shape, and unorganized mycelia	[26,27]
<i>A. niger</i>	3.6 g/L	Conidia	Swelling, aggregates	[60]
<i>B. cinerea</i>	1000 µg/mL	Hyphae	Swelling, abnormal shape, and unorganized mycelia	[26,27]
<i>B. cinerea</i>	1.0, 2.0, 4.0 mg/mL	Hyphae	1 mg/mL, thinner and corrugated surface; 2 and 4 mg/mL, rough surface with depressions and crates	[46]
<i>C. gloeosporioides</i>	3,000 Da and fungicide	Hyphae	Shrink, abnormal, deform, and collapsed	[45]
<i>F. oxysporum</i> f. sp. <i>gladioli</i>	1.0 mg/mL	Hyphae and conidia	Dehydration, contorted hyphae with abnormal shapes	[11]
<i>P. expansum</i>	1000 µg/mL	Hyphae	Swelling, abnormal shape, and unorganized mycelia	[26,27]
<i>P. botryose</i>	0.5 µg/mL	Hyphae and oogonia	Size reduction, whiter pigmentation	[74]
<i>R. stolonifer</i>	1.5 mg/mL	Hyphae	Convuluted	[32]
<i>R. stolonifer</i>	1 mg/mL	Conidia	Alterations in conidia ornamentation	[43]

<i>R. stolonifer</i>	500 µg/mL	Hyphae	Swelling, abnormal shape, and unorganized mycelia	[27]
<i>R. stolonifer</i>	1.0% amended with 0.1% lime essential oil and 0.1% beeswax	Hyphae	Intense dehydration and distorted	[11,65]
<i>T. harzianum</i>	0.01, 0.05, 0.1%	Hyphae	Corrugated surface and formation of granulated material	[77]
<i>S. sapinea</i>	0.01, 0.05, 0.1%	Hyphae	Corrugated surface, formation of granulated material, at highest concentrations of 1%, extracellular surface laying	[77]

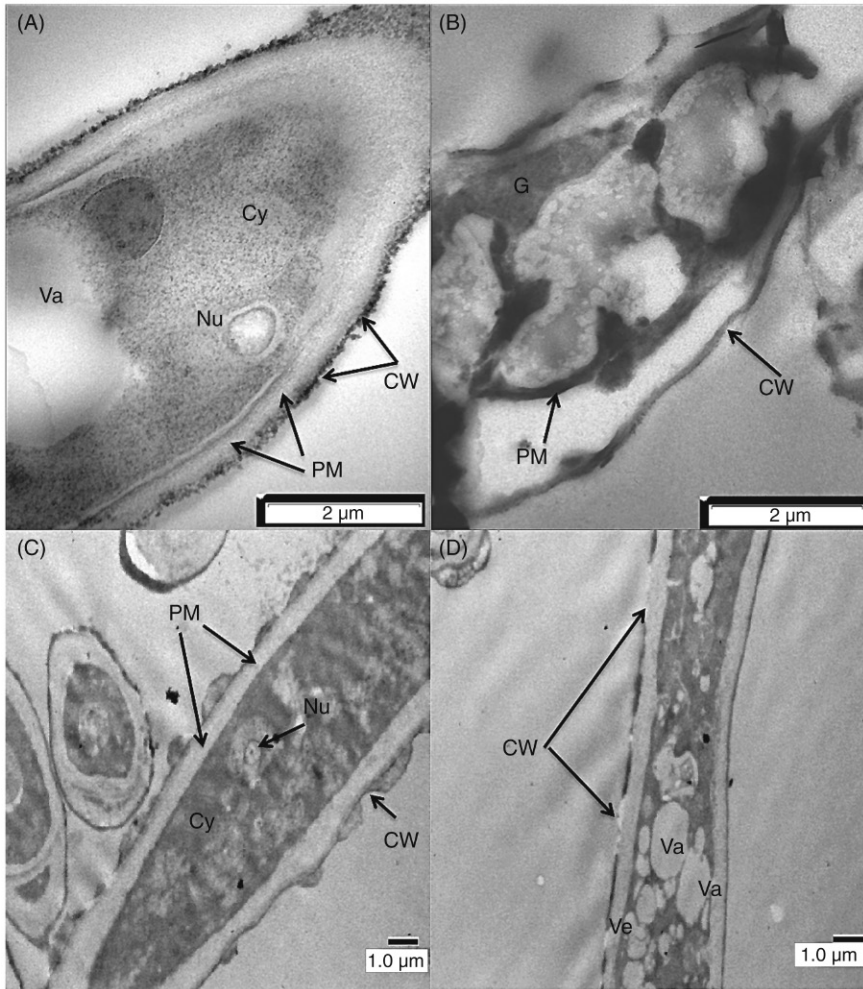


Figure 9.5 Transmission Electron Microographies of Mycelium of *A. alternata* Obtained from Tomato (A, B) and Mango (C, D). Untreated fungus showing mycelium with a well-defined cell wall, plasmatic membrane, and some visible organelles (A, C). Treated fungus with chitosan at 1.5% concentration showing mycelium with a disorganized fungal cell wall, retracted plasma membrane, almost no longer visible and intense granulation; at 1.0%, mycelium with numerous lipid bodies and intense vacuolization. Some areas of the fungal cell wall already disintegrated. CW, cell wall; PM, plasmatic membrane; Va, vacuole; Ve, vesicles; Cy, cytoplasm; G, granules; L, lipid bodies; Nu, nucleus.

material, compared with the intense disintegration of the infected cells in nontreated and inoculated fruit [33,35]. During interaction, examinations of the affected fungus showed significant cellular damage such as, cell wall loosening, cytoplasm disintegration, and lysis. Fungal growth was restricted; therefore, it was not able to invade deeper parenchyma tissues. Similar

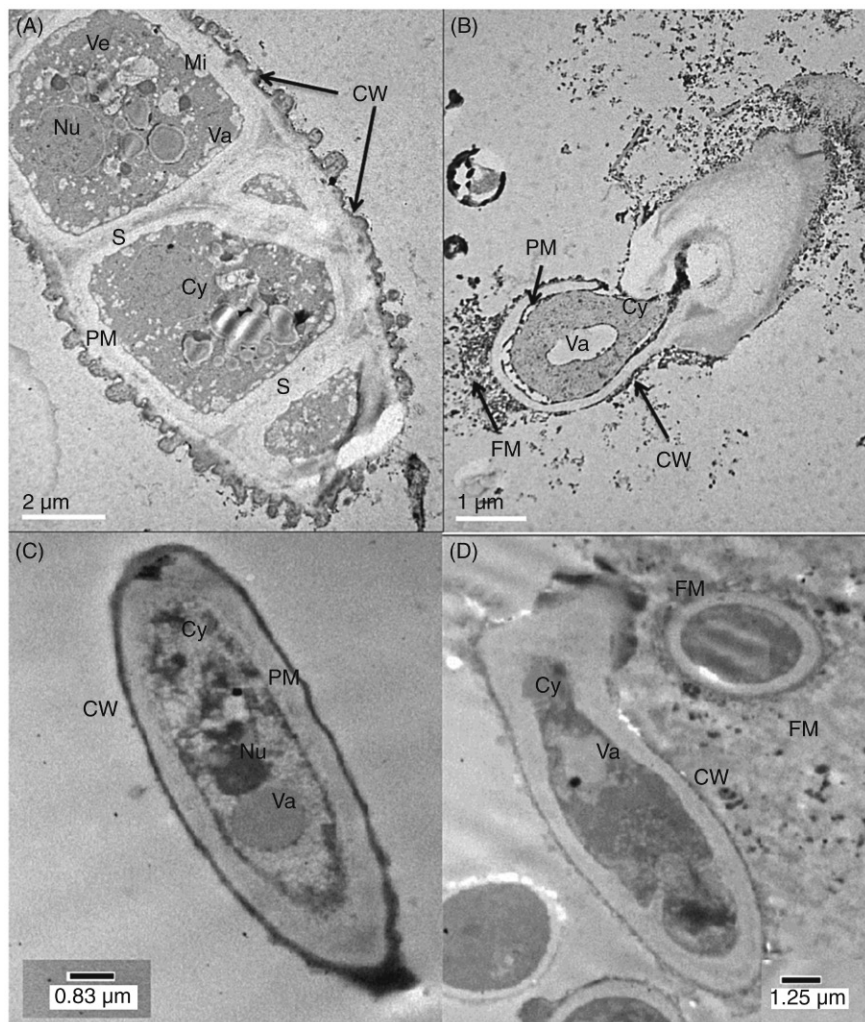


Figure 9.6 Transmission Electron Micrographies of Conidium of *A. alternata* Obtained From Tomato (A, B) and Mango (C, D). Nontreated fungus showing a septate conidium with a well-defined cell wall and plasmatic membrane, containing visible organelles such as nucleus, vacuoles, and vesicles (A, C), while in treated fungus with chitosan at 1.0 and 1.5% concentrations, conidium presented a disorganized fungal cell wall, retracted plasma membrane, and large vacuoles, formation of extra fibrillar material, and visible leakage of the cytoplasm. CW, cell wall; PM, plasmatic membrane; Va, vacuole; Ve, vesicles; Cy, cytoplasm; Mi, mitochondria; Nu, nucleus; FB, fibrillar material.

results were reported for *F. oxysporum* f. sp. *radicis-lycopersici* – tomato roots interaction [16,50]; in this case, the non-chitosan-treated and inoculated plants showed more severe disease symptoms, because this fungus rapidly colonized roots and invaded the whole plant tissue systems. On the contrary,

Table 9.4 Summary of *in vitro* observations of cellular alterations of chitosan-treated phytopathogenic fungi by TEM microscope technique

Fungi	Chitosan concentration	Fungal structure	Cellular alterations	References
<i>A. alternata</i>	1.5% medium molecular weight	Hyphae and conidia	Loosened, broken, and uneven cell walls, cellular lysis	[69]
<i>A. alternata</i>	1% low molecular weight	Hyphae and conidia	Disruption of the cell wall, intense and extended vacuolization, formation of fibrillar material, leakage of cytoplasm	[11,52]
<i>A. niger</i>	0.1% molecular weight of 50 Dka	Conidia	Alteration in the outer cell and nucleus membrane	[53]
<i>C. floridanum</i>	0.5, 1.0, 2.0 mg/mL	Hyphae	At 0.5 mg/mL, retraction of the plasma membrane; at 1.0 mg/mL, disorganized cytoplasm with aggregated material, and at the highest concentration empty cells	[49]
<i>C. destructans</i>	0.5, 1.0, 2.0 mg/mL	Hyphae	At 0.5 and 1.0 mg/mL, signs of disintegration of plasma membrane, increment in vacuole size; at 2.0 mg/mL, disintegration of plasma membrane, few organelles are visible	[49]
<i>F. acuminatum</i>	0.5, 1.0, 2.0 mg/mL	Hyphae	At 0.5 mg/mL, aggregation of cytoplasm; at 1.0 mg/mL, appearance of cell wall like material; at 2.0 mg/mL, disorganized cell and no organelles visible	[49]
<i>F. oxysporum</i>	0.5, 1.0, 2.0 mg/mL	Hyphae	At 0.5 mg/mL, increased vacuolization; at 1.0 mg/mL, disorganized cells, plasma membrane disintegration and no visible organelles; at 2.0 mg/mL, distorted cells	[49]
<i>F. oxysporum</i> f. sp. <i>radicis-lycopersici</i>	1, 2, 3, 4 mg/mL	Hyphae	At 1 mg/mL, intense vacuolation; at 2.0 mg/mL, retraction of plasmalemma and cytoplasm aggregation; at 3.0 mg/mL, cytoplasm reduced to strands electron dense material; at 4.0 mg/mL, distorted cell and highly disorganized	[15]

<i>F. oxysporum</i> f. sp. <i>radicis-lycopersici</i>	0.01 mg/mL	Conidia	Plasmalemma retraction, cytoplasm aggregations, and cell content disorganization	[56]
<i>P. capsici</i>	Oligochitosan at 10, 100 µg/L	Hyphae	At 10 µg/L, cell distortion and absence of plasmalemmasomes; at 100 µg/L, disorganized cell, absence of vacuoles and plasmalemmasomes, thickening of plasmalemma, and appearance of tubular material	[78]
<i>R. stolonifer</i>	1.5 mg/mL	Hyphae	Loosened, irregular, and altered cell walls	[31,32]
<i>T. harzianum</i>	0.01, 0.1, 0.2%	Hyphae	At 0.01%, dense cytoplasmic content, formation of mucilaginous extracellular material, and intracellular lipid bodies; at 0.1% and 0.2% disorganized cytoplasm	[77]
<i>T. harzianum</i>	0.01, 0.1%	Hyphae	At 0.01 loss of cytoplasm integrity; at 0.1%, vesiculation, degeneration of organelles, abundant presence of extracellular material	[71]
<i>S. sapinea</i>	0.01, 0.1, 0.2%	Hyphae	At 0.01%, cell wall “exfoliation”; at 0.1%, cell wall disorganization and granulation; 0.2%, cytoplasmic disintegration and thinning of the cell wall	[77]
<i>S. sapinea</i>	0.01, 0.05, 0.1%	Hyphae	At 0.01%, disruption of the plasma membrane, aggregation of cytoplasm content, and change in its density; at 0.05%, excessive vesiculation of membrane structure, increased vacuolation, and complete disruption of plasma membrane; at 0.1%, complete loss of membrane integrity	[71]

Table 9.5 Summary of the effect of chitosan during the infection process of various plant-pathogen interactions

Plant pathogen interaction	Chitosan concentration/ spore load	Interaction response	References
Tomato fruit – <i>A. alternata</i>	Medium molecular weight chitosan 1.5%/10 ⁶ conidia/mL	Fungal infection took place all over the tissue; significant changes corresponded only to the infection process	[69]
Bell pepper fruit – <i>B. cinerea</i>	10 mg/mL/2 × 10 ⁶ conidia/ mL	Fungus restricted to the parenchyma cells; host cells showing integration and preserved; fungal cells showing intense damage	[33,35]
Cucumber plants – <i>Pythium aphanidermatum</i>	400 µg/mL/10 ⁸ CFU/mL	Deposition of new material; formation of papillae on the root; fungal cells showing serious ultrastructural alterations	[34]
Tomato plant – <i>F. oxysporum</i> f. sp. <i>radicis-lycopersici</i>	1 mg/mL/discs of mycelium	Restriction of fungal growth to the epidermis and outer cortex of the root cells; new intracellular material; fungal cells showing ultrastructural alterations	[16]
Tomato plant – <i>F. oxysporum</i> f. sp. <i>radicis-lycopersici</i>	25.0, 37.5 mg/mL/3 × 10 ⁸ CFU/mL	Fungal growth restricted to the external tissues of the root; accumulation of new material and tissue; hyphae suffered dramatic morphological and ultrastructural alterations	[50]
Tomato plant – <i>B. pumilus</i> – <i>F. oxysporum</i> f. sp. <i>radicis-lycopersici</i>	1 mg/mL/10 ⁶ cells/mL/3 plugs of 5 mm diameter	Fungal growth restricted to external root tissues, formation of structural barriers, and formation of new materials	[17]
Pea pod plant – <i>F. solani</i>	180-mesh chitosan/3 × 10 ⁶ conidia/mL	Hyperaccumulation of chitosan and polymeric material in the adjacent fungal cells	[40]
Lemon fruit – <i>P. digitatum</i>	1 mg/mL/1 plug of 1 mm diameter	Growth restriction of <i>P. digitatum</i> ; disorganized and altered hyphae structure	[18]

the colonization pattern of the chitosan-treated plants showed that in general *F. oxysporum* f. sp. *radicis-lycopersici* was kept in the outer epidermal cells of the roots, and in some cases in cells of the outer cortex with concomitant serious morphological and ultrastructural changes of the pathogenic fungus such as cytoplasm disorganization, vacuolation, and retraction of plasma-lemma. In other light and TEM microscopy studies of the integration of a bacterial antagonist, *Bacillus pumilus*, with chitosan [18], it was seen that growth of *F. oxysporum* f. sp. *radicis-lycopersici* was restricted to the epidermis associated with densely stained appositions on the cell wall, formation of new opaque material, and accumulation of polymorphic droplets in the host cells. In other interactions between peapod tissues and *Fusarium solani* f. sp. *pisi* [40], histological localization of chitosan showed a dense accumulation of hexosamine polymers within the adjacent infected cells. SEM examinations of the infection process of *Penicillium digitatum* in lemon fruits treated with chitosan showed plasmolyzed and collapsed hyphae with lack of spore formation. In addition, light micrographs of fungal hyphae showed high vacuolation, distortion, and in extreme cases they were empty. TEM observations also confirmed fungal growth restriction coinciding with disorganized and distorted cells of the host exocarp tissue in contact with the fungus, which in turn was severely damaged [18].

CONCLUDING REMARKS

A number of studies confirmed the *in vitro* fungicidal effect of chitosan on various phytopathogenic fungi grown on nutrient media amended with different concentrations of this polymer. To understand the extent of damage of chitosan on fungi, scientists have conducted studies at microscopic level that allow examining in detail the fungal response to this compound under controlled conditions. In this regard, studies on the morphology and ultrastructure of the chitosan-treated fungi have been carried out by using conventional optical/light microscopy and other advanced techniques including SEM, TEM and confocal microscopy [11]. According to the literature review, the application of chitosan results in serious cellular degeneration from the surface to the inner structures of numerous fungal genera, including *Alternaria*, *Botrytis*, *Fusarium*, and *Rhizopus*, in which cellular destruction increased as chitosan concentration increased. As it has been mentioned in this review, one of the confirmed modes of action is the electrostatic interaction between chitosan and the microorganism [47,51,61], which has been strongly associated with dramatic cell wall injuries, noted

by observations presenting consistent and frequently signs of damage, such as surface distortion, swelling, alterations in shape, and in some cases loss of its integrity leading to leakage of the cytoplasmic content. The fungal cell wall is the first organelle in direct contact with chitosan, and it is particularly affected due to the anionic characteristic of the cell wall and its inability to be repaired as may also occur with other stressors. At high magnifications, it was also noted that fungal plasma membrane of most of the chitosan-treated phytopathogenic fungi was seriously disturbed, mainly showing detachment from the cell wall, shrinkage, and in extreme cases disappearance. Another widely observed structural damage found in the treated fungal cell is the progressive number of lipid vacuoles that develop (vacuolation). Examinations showed that vacuoles start to grow, progressively occupying larger areas of the cytoplasm. According to Henicas and Wheatley [42], this phenomenon may correspond to an adaptive “physiological responses” to external agents, in this case to chitosan application. Functions of the fungal cell vacuole include intracellular homeostatic and regulation of many enzymatic activities [20]. Therefore, we may consider chitosan a “vacuolating” agent. To date, ultrastructure observations have also revealed unusual cytoplasmic granulation in the cells of chitosan-treated fungi. To our knowledge, no description of such granules in the chitosan-treated fungal cell cytoplasm has been reported, but in previous studies [76] similar granulation was observed in the cytoplasm of the hyphae of *F. oxysporum* f. sp. *vasinfectum* treated with the plant defensin NaD1 from the flowers of *Nicotiana glauca* after binding the fungal cell wall. Further cytochemical evaluations would be beneficial, including specific stains to elucidate its composition and function. Development of extracellular mucilaginous-type material on some fungal cell walls has also been observed as a response to chitosan application [69,71,77]. A hypothesis for this characteristic is associated with a stress condition; however, additional studies should be carried out to identify its origin and composition.

In general, studies of the fungal-plant interaction have shown a significant disease reduction in the infected host when treated with chitosan. The induction of resistance through the formation of certain structures that impede the entrance and dissemination of fungi inside the host has been documented. Those studies reported that, in other pathosystems, chitosan induced, among others things, physical changes that limited the penetration and development of the fungi in the host tissues, with the most notable activity being the formation of structural barriers through accumulation of new depositions phenol, lignin-like, inter- and intracellular material

[17,18,34,35,39]. As for the fungal interaction, structural alterations were similar to those reported for *in vitro* studies, demonstrating that chitosan has twin properties: fungicidal and eliciting.

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PART 3

Biochemical and Molecular Aspects of Chitosan

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CHAPTER 10

Biochemical Aspects of the Chitin Fungicidal Activity in Agricultural Uses

Cristóbal Lárez Velásquez, Maura Rojas Pirela

INTRODUCTION

Chitin is one of the oldest organic materials in the world. It has been found in fossils of trilobites — a group of extinct marine arthropods — which appear to date back about 375–500 million years. The conscientious use of chitinous materials by humans is more recent. It has been reported that such materials were used as wound healing accelerants by Koreans, who have long used the pen of the squid as a source for chitin in abrasion treatments, and by Mexicans, who have traditionally employed the chitosanaceous cell walls of mushrooms to accelerate the healing of machete gashes [1].

Agriculture has been another area in which humans have employed chitinous materials, especially in the pursuit of applications for rather cheap materials and, simultaneously, solving environmental problems caused by their increasing accumulation in processing plants of seafood products. Thus, these materials, especially some of their specific derivatives, show beneficial effects as soil amendments [2,3], coating for seed and fruit preservation [4,5], germination stimulant [6,7], fungicides [8,9], plant growth inducers [10], and nematicides [11].

Since the mid-twentieth century, users began to notice the positive effects of chitin soil amendment in decreasing some fungal diseases in plants [12–14]. Table 10.1 provides a brief list of phytopathogen microorganisms associated with fungal diseases in which chitin has demonstrated beneficial effects for decreasing the incidence of their associated diseases. Unfortunately, reported results are not always positive and some studies have indicated that, under some conditions of application, phytotoxicity can appear [15,16]. It has been assumed that phytotoxicity can be caused by the relatively narrow C:N ratio of the chitin polymer structure (6:4), similar to that observed with other nitrogenous amendments [15]. Some studies showing contradictory results related to the effectiveness of chitin amendments on the control of phytopathogen have also been reported [17,18].

Table 10.1 Phytopathogenic fungi and caused diseases whose incidence is reduced by application of chitin

Fungus	Disease	Application method	References
<i>F. solani</i> f. sp. <i>phaseoli</i>	Root rot of beans	Addition of small quantities of chitin to soil	[12]
<i>F. oxysporum</i> f. sp. <i>conglutinans</i>	Vascular wilt of radish	Addition of small quantities of chitin to soil	[12]
<i>F. oxysporum</i>	Celery yellows	1 kg chitin (Sigma Chemical Co.) per experimental unit (single beds, 6 m long, with 1 m between bed centers) was uniformly spread over the bed tops and incorporated into the soil to a depth of 15–20 cm using a buttonwillow mulcher just prior to transplanting	[19]
<i>R. solani</i> and <i>F. solani</i>	Root infection of sunflower	Soil amendment with crustacean chitin used alone or with <i>P. aeruginosa</i> or <i>B. subtilis</i>	[20]
<i>M. phaseolina</i>	Root infection of chickpea	Soil amendment with crustacean chitin used alone or with <i>P. aeruginosa</i> or <i>B. subtilis</i>	[20]
<i>M. phaseolina</i> , <i>R. solani</i> , <i>F. oxysporum</i> , and <i>F. solani</i>	Root infection of chili	Application of <i>P. aeruginosa</i> or <i>Paecilomyces lilacinus</i> alone or with crustacean chitin	[21]
<i>Plasmodiophora brassicae</i>	Clubroot formation in Chinese cabbage	Individual cabbage seedlings were transplanted into pots (3500 mL) containing a mixture of 3% chitin compost and 50 mL of chitin broth (T1) or the same quantity control compost and control compost broth (T2); the media in each pot were then infected with <i>P. brassicae</i> ; the population of chitinase-producing bacteria in T1 was larger than that observed in T2	[22]

<i>M. fijiensis</i>	Black Sigatoka in banana and plantain	Application of foliar substrates (a mineral solution plus urea, barley flour, and colloidal chitin) allowed to decrease fungicide applications 43–46%, when sprayed in rotation with conventional fungicides	[23]
<i>Penicillium expansum</i>	Postharvest blue mold rot in pear fruit	Cell-free filtrate of chitin-supplement culture media in which yeast was incubated for 24 h exhibited antifungal activity against <i>P. expansum</i> in pear fruit wounds	[24]
<i>R. solani</i>	Damping off of pepper	During planting of pepper seeds (pots 12 cm in diameter), 20 ml of King's medium B containing 0.5% of chitin were added	[25]
<i>C. falcatum</i>	Sugarcane red rot	The influence of chitin on antagonistic activity of microbial strains against <i>C. falcatum</i> was studied by dual culture technique on oatmeal agar in the presence or absence of chitin	[26]

The present review focuses on the analysis of the main mechanisms proposed to account for the beneficial effects of chitin in reducing fungal diseases of crops. Additionally, some actions that may be implemented in the near future to take advantage of the knowledge acquired on the different mechanisms of protection of plants triggered by chitinous materials are briefly discussed.

SOME MECHANISMS PROPOSED FOR THE FUNGICIDAL ACTIVITY OF CHITIN

In order to attempt to explain the beneficial effects of chitin in decreasing fungal diseases in plants, the following modes of action have been proposed: (i) occurrence of a fertilizer effect, (ii) indirect inhibition of the pathogens via their decomposition byproducts (i.e., ammonia released during chitin decomposition) [27], (iii) growth promotion of antagonist microorganisms, (iv) stimulation/support of fungicide-producing microorganisms [19,26,28], (v) mycorrhiza growth stimulation [29] and (vi) elicitor activity of chitin [30]. An overview of the different proposed mechanisms of action is reported in Figure 10.1.

Fertilizer Effect

Chitin decomposition by-products such as ammonia may become a source of slow but sustained release of nitrogen, which promotes growth of plants, making them healthier and less susceptible to fungal attacks as observed in

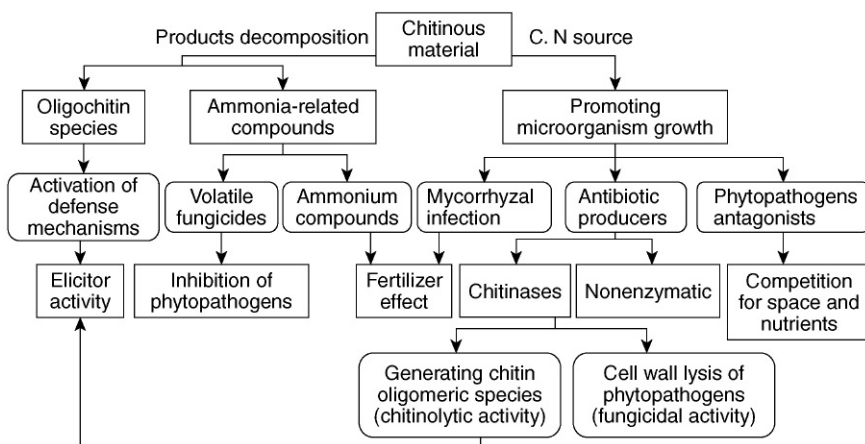


Figure 10.1 A Schematic Representation of the Proposed Mechanisms for the Antifungal Activity of Chitinous Materials.

the fast greening of celery transplants in plots amended with chitin. However, in this study, fresh weight of marketable celery stalks was not significantly different as compared to treatments without chitin. It is important to emphasize that size of individual plants did not increase in chitin-amended plots, but yield was increased by the reduction in yellows incidence caused by *Fusarium oxysporum* [19].

Indirect Inhibition of Phytopathogens

The fungicidal activity of some by-products from chitin decomposition in soil has also been linked to the so-called volatile fungistatic factor [27,31], specifically associated with ammonia, although other volatile by-products such as methylamine and ethylamine cannot completely be excluded [27]. Ammonia-related compounds may be released during the chitin degradation in soil by different soil-borne organisms [32].

Stimulation/Support of Growth of Profitable Microorganisms

It has been demonstrated that soil amendments with chitin can result in significant control of root-infecting microorganisms, changing the soil microflora by increasing antagonist microorganisms (which operate by competition for food and space, hyperparasitism, hyphal lysis, etc.) around the roots [33,34]. Therefore, the increase of existing antagonists in the root environment by using chitin amendment is an advisable strategy in the biological control of pathogens [35]. Furthermore, if chitin-containing amendment is added with a suitable antagonist, it should produce more reliable results as demonstrated by the use of chitin amendments with *Pseudomonas aeruginosa* in suppressing *Macrophomina phaseolina*, *Rhizoctonia solani*, *F. oxysporum* and *Fusarium solani* attacks on the roots of chili (*Capsicum annuum* L.) [21]. Interestingly, crustacean waste powder showed a more positive effect on plant growth than pure chitin used alone or with microbial antagonists. Presumably, other nitrogenous materials present in the waste may provide an additional food base for saprophytic and introduced bacteria, resulting in a higher positive effect on plant growth. Moreover, chitin can also be effective in reducing usage of chemical fungicide when applied as a foliar substrate to encourage native bacterial population with lytic activity on pathogens that attack the aerial parts of the plants. Thus, Patiño et al. [23] have found that the fungus *Mycosphaerella fijiensis* Morelet, which causes the Black Sigatoka disease in banana (*Musa paradisiaca* L.) and plantain (*Musa acuminata* Colla), can be controlled with application of foliar substrates containing colloidal chitin, decreasing conventional fungicide applications by about 43–46%, when sprayed in rotation.

Biocide-producing microorganisms operate by producing various secondary metabolites that include mainly cell-wall lytic enzymes and other types of antibiotics compounds and include the following:

- *Enzymatic biocides*: Although many researchers agree that chitinolytic activity of antagonist microorganisms does not always have a direct relationship with their fungicidal activity [36,37], stimulating the growth of microorganisms that produce chitinolytic enzymes is one of the action mode most often proposed (Table 10.2). The chitinolytic activity of the enzymes produced by some microorganisms has been clearly demonstrated by Malathi and Viswanathan [26] during studies on biocontrol of the fungus responsible for sugarcane red rot (*Colletotrichum falcatum*) with either fungal (*Trichoderma* spp.) or bacterial (*Pseudomonas fluorescent*) antagonists. By using a cell-free culture filtrate of them, at different concentrations, they established that pathogen growth is inhibited, with inhibition efficacy increasing when the filtrate concentration is increased. It was also confirmed during *in vivo* studies that chitin had influence in suppressing the pathogen growth.
- *Nonenzymatic biocides*: Isolation and characterization of nonenzymatic antifungal compounds during the growth of some bacteria in chitin-rich media has also been reported. Thus, San-Lang et al. [38] isolated and elucidated a complex polysaccharide, named “pafungin,” during growth of *P. aeruginosa* in a shellfish waste-containing medium, which was effective against all 39 fungi strains tested. Similarly, during the growth of *Bacillus subtilis* in a chitin-containing medium, the production of nonenzymatic compounds reportedly displayed antifungal activity on *F. oxysporum* [48].

Some works that initially referred to the positive effects of chitin on mycorrhizal colonization, including studies on tomato cultivation [49] that showed favorable effects from adding chitin and derivatives, and the positive effects of chitin on the length of extra-radical mycelium (ERM) growth reported by Gryndler and Vosatka [50], which emphasized that complexity and variability of responses followed by addition of organic amendments to the growth substrate are factors that must be taken into consideration when examining plant-substrate-arbuscular mycorrhizal fungi (AMF) interactions. Chitinous materials, as crustacean shellfish waste, also demonstrated a stimulatory effect on the AMF colonization of strawberry (*Fragaria ananassa*) [29]. Simultaneously, Staehelin et al. [51] demonstrated that *N*-acetyl groups in lipooligosaccharides of chitin (i.e., β -1,4-linked oligomers of *N*-acetyl-D-glucosamine), with a fatty acid replacing the *N*-acetyl group on

Table 10.2 Some chitin-stimulated microorganisms producing fungicides with chitinolytic activity

Microorganism	Type	Activity exhibited	References
<i>P. aeruginosa</i> K-187	Nonenzymatic (complex carbohydrate with MW 66 kDa, which contains 17% of amino acids, and 62% of carbohydrate	Culture broth of <i>P. aeruginosa</i> K-187 retained more than 90% of its inhibitory activities to <i>F. oxysporum</i> even after being treated at a pH of 3 or 100°C for 30 min	[38,39]
<i>Alcaligenes xylosoxydans</i>	Chitinase	Growth of <i>Fusarium</i> sp. and <i>Rhizoctonia bataticola</i> was inhibited 74% and 81%, respectively, by culture filtrate	[40]
<i>Monascus purpureus</i> CCRC31499	Chitinase and protease	Spore germination and germ spore elongation of <i>F. oxysporum</i> were inhibited by the supernatant from cultured media supplemented with chitin waste	[41]
<i>Pseudomonas</i> sp., <i>Pantoea dispersa</i> , and <i>Enterobacter amnigenus</i>	Chitinase and protease	In vitro, these strains could inhibit the growth of <i>Fusarium</i> sp. and <i>M. phaseolina</i> ; the culture filtrate inhibiting hyphal elongation was observed microscopically	[42]
<i>B. subtilis</i> W-118	Exochitinase (MW 20.6 kDa and a pI = 6)	Growth of <i>F. oxysporum</i> was 100% inhibited after incubation for 1 day with sterilized chitinase solution (5.6 units/mL)	[43]
<i>Pseudomonas fluorescens</i>	Chitinase (MW 43 kDa)	Mycelial growth inhibition of <i>F. oxysporum</i> f. sp. <i>dianthi</i> causing vascular wilt of carnation	[44]

(Continued)

Table 10.2 Some chitin-stimulated microorganisms producing fungicides with chitinolytic activity (*cont.*)

Microorganism	Type	Activity exhibited	References
<i>B. thuringiensis</i> subsp. <i>aizawai</i>	Chitinase (66 kDa) significantly inhibited by K ⁺ and EDTA and completely inhibited by Hg ²⁺	Purified chitinase showed lytic activity against cell walls from six phytopathogenic fungi and inhibited the mycelial growth of <i>Fusarium</i> sp. and <i>Sclerotium rolfsii</i> ; the biocontrol efficacy of the enzyme was tested in the protection of bean seeds infested with six phytopathogenic fungi	[45]
<i>B. subtilis</i> NPU 001	Chitinase (MW 31 kDa and a pI = 5.4)	- Hyphal extension of the fungus <i>F. oxysporum</i> was inhibited - Production of chitotriose as the major enzymatic hydrolysate from colloidal chitin	[46]
<i>B. laterosporous</i> Lak1210	Two chitinase (a 89.6 kDa four-domain chitodextrinase and a 69.4 kDa two-domain called ChiA1)	Fungus <i>Fusarium equiseti</i> was indeed inhibited by the enzymes in a dose-dependent manner	[47]

their nonreducing end, are necessary for their bioactivity as signal molecules excreted by Rhizobia (generally known as Nod factors) during the establishment of the legume-Rhizobia symbiosis [52,53]. Consequently, chitin oligomers could be provided as a precursor substrate for these metabolites. On the other hand, Gryndler et al. [54] showed that chitin added to sand-soil-based cultivation substrates stimulated the root colonization, growth of ERM, and production of spores of AMF using *Allium ampeloprasum*, *Plantago lanceolata* and *Lactuca sativa* as host plants. Interestingly, stimulation of AMF sporulation was also observed when autoclaved mycelium of *F. oxysporum*, which contain chitin, was used instead of chitin.

It is important to realize that promotion of microorganisms favoring mycorrhizal infection by chitin would produce similar and indistinguishable results from those generated by a direct fertilizer effect.

Elicitor Activity of Chitin

Plants have the ability to recognize molecules arising from pathogens. These molecules can elicit immune responses in host plants and are referred as pathogen-associated molecular patterns (PAMPs), or more broadly as microbial-associated molecular patterns (MAMPs). They appear to possess a chemical pattern, absent on host molecules, enabling them to detect the presence of potential invading pathogens. Some MAMPs include peptidoglycans from Gram-positive bacteria, lipopolysaccharides of Gram-negative bacteria, eubacterial flagellin, glucans, and proteins [55].

Chitin is a current polysaccharide in the cell walls of fungi. When suffering fungal attacks, plants release chitinolytic enzymes that degrade exogenous chitin, generating fragments of it. Chitin-oligomeric species have been recognized as one of the MAMPs derived from the cell walls of fungi. Thus, *N*-acetylchitoooligosaccharides are typical fungal MAMPs that trigger various defense responses in both monocots and dicots, indicating the presence of a conserved machinery to perceive these oligosaccharides in a wide range of plant species [56,57] and, according to a more recent finding, also in animals [58].

It is well established that chitin oligosaccharides induce different plant protection reactions such as (i) callose deposition on mature parts of the *Arabidopsis* roots [59]; (ii) increasing activity of lignification-associated enzymes in cultured carrot cells [60]; (iii) lignification in wounded wheat leaves [61]; (iv) activation of lignification-related enzymes in leaves of soybean [62]; (v) expression of defense-associated genes [63] (i.e., enhancing rice plant resistance against rice blast fungus, *Magnaporthe grisea*, after pretreatment with

N-acetylchitooligosaccharides) [64]; (vi) production of reactive oxygen species (ROS) in rice cell [65] and (vii) activation of mitogen-activated protein (MAP) kinases in plants as *Arabidopsis* [66].

Likewise, increased susceptibility of *Arabidopsis* to the powdery mildew pathogen *Erysiphe cichoracearum* after mutation with loss of function of the chitin-oligomers responsive gene [67] can be considered a confirmation of these reactions.

In order to perform some of their preventive functions, plants have developed mechanisms related to MAMPs detection, collectively referred as pattern recognition receptors (PRR). Plants possess specific chitin-PRR to sense and defend themselves from phytopathogenic fungi and phytophagous insect attack. In the latter case, chitin-binding lectins have also been shown to possess insecticidal activity [68].

Some studies related to chitin receptors in plants include the following:

1. A small-sized lectin exhibiting chitin-binding specificity was isolated from the rhizomes of stinging nettle [69]. Later, it was found to inhibit the growth of several phytopathogenic and saprophytic chitin-containing fungi *in vitro* but its antifungal action differs from that of chitinases, even if it showed a synergistic action with a chitinase to inhibit fungal growth [70].
2. Specific perception of subnanomolar concentrations of chitin fragments by tomato cells was reported by Felix et al. [71], establishing that oligomers with four or more *N*-acetyl-D-glucosamine residues have significant elicitor effects at concentrations below 10 pM.
3. Chitin elicitor-binding protein, whose existence as a high-affinity binding site for chitin on microsomal membrane preparation from suspension-cultured rice cells, was proposed for the first time by Shibuya et al. [72]. It is a plasma membrane glycoprotein containing two extracellular lysin motifs (LysM) that bind chitin directly [73].
4. A receptor-like kinase, designated CERK1, which is essential for chitin elicitor signaling in *Arabidopsis* has been described by Miya et al. [74] as a plasma membrane protein containing three LysM in the extracellular domain and an intracellular Ser/Thr kinase domain with autophosphorylation/myelin basic protein kinase activity, pointing to CERK1 plays a critical role in fungal MAMP perception in plants.

As a final comment to this section, it is important to highlight that fungi have evolved some mechanisms to outwit plants by developing specific molecules. These effector proteins bind to PAMPs and hide them from the plant receptors. It has been demonstrated that these effectors contain similar

molecular arrangements to those in plant receptors to recognize PAMPs (i.e., LysM) [75,76].

FUTURE TRENDS

Progress in genetic engineering and molecular biology now allows identification and separation of DNA-microbial sequences capable of expressing a large number of molecular species – including enzymes – that can be employed for producing a chemical compound from the desired gene on a large scale in the bacterial cell and obtaining it by secretion outside the cell. Genetically modified *Bacillus thuringiensis* no. 4.1 is an example of such microorganisms. A DNA fragment from it, encoding for 598 amino acids of a chitinase protein, was subcloned into the pQE-30 expression vector and transformed into *Escherichia coli* M15 (pREP4). The recombinant enzyme hydrolyzed *N*-acetylchitooligosaccharides mainly to *N*-acetylchitobiose and was active toward chitin, carboxymethyl-chitin, colloidal chitin, glycol chitin, and 4-methyl-umbelliferyl- β -D-*N,N*-diacetylchitobiose but did not hydrolyze *N*-acetyl-chitobiose, suggesting that the enzyme does not possess *N*-acetylglucosaminidase activity [77].

More recently, a method for producing chitinase with fungicidal effects in a variety of phytopathogenic fungi has been patented by using the SEQ ID 7 sequence in *B. subtilis* 128 strain, which acts as coding for this chitinase [78]. For the purposes of its overproduction, *E. coli* is indicated as host cell. Table 10.3 shows a brief list of works related to overexpression of chitinases with fungicidal activity.

On the other hand, and even though it is a controversial subject, techniques using genetic manipulations of the *chit42* gene from *Trichoderma harzianum* have been assayed for improving plant resistance to fungal pathogens. This gene, encoding an active endochitinase exhibiting a strong antifungal effect, has been introduced into genomes of tobacco (*Nicotiana tabacum* L.), apple tree (*Malus domestica*), and potato (*Solanum tuberosum*), and when inserted with a constitutive promoter undergoes continuous expression. These transgenic plants are characterized by considerably higher resistance to fungal pathogens [91]. It should also be possible to obtain plants overexpressing receptors, including chimeric receptors, to improve protection and/or resistance spectrum, such as the component of the chitin receptor for CERK1 (LysM) in *Arabidopsis thaliana* with either intracellular or extracellular region from another appropriate receptor that allows enhancing the resistance to a broad spectrum of fungi and pathogens.

Table 10.3 Some reported studies related to overexpression of chitinases on various microorganism

Microorganism	Cloned Gen/DNA sequence	Expressed protein	Physicochemical properties	Heterologous organism	References
<i>Chromobacterium violaceum</i>	ATCC 12472 chitinase gen of <i>C. violaceum</i>	Chitinase protein secreted (CvChi45) of <i>C. violaceum</i>	Molecular mass: 43.8 kDa Optimum pH: 5.0 Optimum temperature: 40°C Kinetic parameters specific activity: 22,26 mU/mg	<i>E. coli</i>	[79]
<i>Serratia marcescens</i>	Hit62 chitinase gen of <i>S. marcescens</i>	Chitinase protein (Chit62) of <i>S. marcescens</i>	Molecular mass: 64 kDa Optimum pH: 6.0 Optimum temperature: 55°C Kinetic parameters Km: 3.30 mg/mL Vmax: 0.92 units	<i>B. subtilis</i>	[80]
<i>Chitiniphilus shinanonensis</i>	Operon of genes encoding putative chitinolytic enzymes (chicCDEFG) of <i>C. shinanonensis</i>	Chitinases proteins rChiC, rChiD, rChiE, rChiF, rChiG of <i>C. shinanonensis</i>	Molecular mass: 63–80.8 kDa Optimum pH: 5 Optimum temperature: 43–55°C	<i>E. coli</i>	[81]
<i>Bacillus licheniformis</i>	The gene <i>chi</i> of <i>B. licheniformis</i> DSM13	Chitinase protein (GH18) of <i>B. licheniformis</i> DSM13	Molecular mass: 65 kDa Optimum pH: 7.5 Optimum temperature: 70°C	<i>Lactobacillus plantarum</i>	[82]
<i>N. tabacum</i> and <i>A. thaliana</i>	Chitinase gen of <i>N. tabacum</i> and <i>A. thaliana</i>	Protein chitinase secreted of <i>N. tabacum</i> (NtChiV) and <i>A. thaliana</i> (AtChiC)	pI NtChiV: 8.84 AtChiC: 9.08 Enzymatic activity NtChiV: 7.53 μ M/min/mg AtChiC: 18.4 μ M/min/mg	<i>E. coli</i>	[83]

<i>Thermomyces lanuginosus</i>	Chitinase gene of <i>T. lanuginosus</i>	Protein chitinase secreted of <i>T. lanuginosus</i>	Molecular mass: 42 kDa Optimum pH: 6.5 Optimum temperature: 60°C Kinetic parameters Km: 0.147 mM Vmax: 814 mmol/min/mg	<i>Saccharomyces cerevisiae</i>	[84]
<i>Sulfolobus tokodaii</i>	ORF BAB65950 from <i>S. tokodaii</i>	Chitinase protein of <i>S. tokodaii</i>	Molecular mass: 77 kDa Optimum pH: 2.5 Optimum temperature: 70°C Enzymatic activity: 75 mU/mg Km: 6.59 mg	<i>E. coli</i>	[85]
<i>Trichoderma asperellum</i>	Chitinase gen (Tachi1) from <i>T. asperellum</i>	Protein chitinase (Tachi1) from <i>T. asperellum</i>	Molecular mass: 44 kDa Optimum pH: 5.5 Optimum temperature: 55°C Enzymatic activity: 17.93 U/mL	<i>Pichia pastoris</i>	[86]
<i>Glaciozyma antarctica</i>	Gene encoding of cold-adapted chitinase (CHI II) from <i>G. antarctica</i> PI12	Protein chitinase (CHI II) secreted from <i>G. antarctica</i> PI12	pI: 9.65 Molecular mass: 39 kDa Optimum pH: 4 Optimum temperature: 15°C Vmax: 3.559 $\mu\text{mol}/\mu\text{g/h}$ Km: 27.918 mg/mL Kcat: 0.915 s ⁻¹	<i>Pichia pastoris</i>	[87]
<i>Ananas comosus</i>	Thermostable chitinase gene of pineapple (<i>A. comosus</i>)	Thermostable chitinase protein of pineapple (<i>A. comosus</i>) (PLChiA)	Molecular mass: 27.8 kDa Optimum pH: 3.0 Optimum temperature: 70°C	<i>E. coli</i>	[88]

(Continued)

Table 10.3 Some reported studies related to overexpression of chitinases on various microorganism (*cont.*)

Microorganism	Cloned Gen/DNA sequence	Expressed protein	Physicochemical properties	Heterologous organism	References
<i>Pochonia chlamydosporia</i>	cDNA sequence of <i>pcchi44</i> via reverse transcription polymerase chain reaction	Extracellular endochitinase	pI: 9.65 Molecular mass: 44 kDA Optimum pH: 6 Optimum temperature: 50°C Vmax: 8.37×10^{-3} mM/s ⁻ Km: 9.65 mM	<i>E. coli</i> BL21.	[89]
<i>Pteris ryukyuensis</i>	A cDNA encoding PrChi-A via rapid amplification of cDNA ends and polymerase chain reaction	Chitinase-A (PrChi-A) with two N-terminal LysM domains and one C-terminal catalytic domain	pI: 3.5 Molecular mass: 42 kDa, Optimum pH: 3 Optimum temperature: 50°C Enzymatic activity: 293.7 U/mg	<i>E. coli</i>	[90]

CONCLUDING REMARKS

Chitinous materials currently possess great comparative advantages for their use as raw material on various processes related to biocontrol of agricultural pests and diseases, mainly because they will continue to be obtained at low cost, in addition to being renewable natural products. Regardless of their use as raw materials for the production of chitosan, such materials can be used with little processing as soil amendments.

The production of small molecular species during soil decomposition may result in various mechanisms promoting plants health, such as (i) a fertilizer effect associated with a nitrogen source, (ii) fungistatic effect due to the volatile fraction being released under certain conditions, and (iii) induction of defense mechanisms in plants promoted by the elicitor activity of oligochitins. Additionally, these materials can also generate a bioactivity favorable toward plants and the growth of beneficial microorganisms, which can be in some cases symbiotic for plants (mycorrhizae) or antagonists for their pathogens, especially fungi and nematodes.

Finally, thanks to the formidable progress in genetic engineering and molecular biology, chitinous materials can be employed to encourage the growth of biocide-producing microorganisms from which can be produced, especially by overexpression recombinant on a large scale, molecular species with controlled biocidal activity (enzymatic and nonenzymatic).

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CHAPTER 11

Chitosan and Changes in Gene Expression During Fruit–Pathogen Interaction at Postharvest Stage

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INTRODUCTION

Importance of Postharvest Losses

Total fruit production in the world has been estimated at 392 million tons, and 30–40% of total agricultural production in developed countries is spoiled due to a lack of adequate postharvest handling prior to consumption [1]. In the case of developed and developing countries, losses of fruits and vegetables are estimated to be about 5–30% and 20–50%, respectively [2].

Perishable fruits and vegetables facilitate microorganism attack due to high water activity and spoil rapidly. Improper postharvest handling, storage, and conservation techniques and deterioration caused by microorganisms increase postharvest losses in fruits and vegetables by up to 40% [3].

Diseases are the leading cause of postharvest losses in tropical and subtropical fruits. The most important decay-causing organisms are *Colletotrichum gloeosporioides* (causal agent of anthracnose), *Diplodia natalensis* (causal agent of stalk rot), *Ceratocystis paradoxa* (causal agent of black rot in banana [*Musa sapientum*] and pineapple [*Ananas comosus*]), and the genera *Penicillium* and *Fusarium* (causal agents of brown rot in pineapple). Anthracnose is the main postharvest problem in various tropical fruits, and latent infections commonly occur in developing fruit before harvest [4].

Mexico is one of the leading producers and exporters of tropical and subtropical fruits [5]. However, these fruits are affected in the postharvest stage by various pathogens, including *C. gloeosporioides*, which cause significant damage to the fruit, hampering their marketing and resulting in

considerable losses [6–9]. To date, fungicides are the only control method, but they are costly and pollute the environment [10–12].

Chitosan is a natural, biodegradable, and nontoxic biopolymer with several antifungal properties. Also, as a resistance inducer, it activates enzymes and genes that encode proteins related to the defense system of the fruit against infection by microorganisms [13,14]. Because of this, chitosan has been proposed as an alternative system in the control of postharvest microorganisms, with fungicidal characteristics and defense mechanism-inducing properties. Induced resistance is a form of active defense involving differential expression of genes and metabolic changes that occur as a result of a specific recognition process between plant and pathogen [15].

Chitosan Properties

Chitosan is a natural, biodegradable, nontoxic, and bioactive polymer, which has shown fungicidal effects against certain plant pathogens and the ability to induce defense mechanisms in plant tissues [16–18]. At the postharvest stage, chitosan is one of the most promising products for the control of *Alternaria alternata* in mango (*Mangifera indica*) [14], *Rhizopus stolonifer* and *Botrytis cinerea* in tomato (*Lycopersicon esculentum*) [19–21], and *Monilia laxa* in cherry (*Prunus avium*) [22], among others.

Mechanisms of Action of Chitosan

The mechanisms of action by which chitosan exerts fungicidal activity have not been fully elucidated. However, some mechanisms have been proposed to explain specific actions, one of which indicates that the electrostatic interaction between NH_{3+} groups of chitosan and phosphoryl groups of the phospholipids present in the cell membrane of fungi cause damage to their physiology and biochemistry, leading to permeabilization of the plasma membrane [23–26]. The authors proposed that oligochitosan exerts antifungal activity by penetrating the cell membrane and binding to intracellular DNA and RNA and alter their vital functions [27]. In addition to its interaction with DNA, for example, it may inhibit mRNA transcription and protein synthesis [28], and its chelating effect may reduce the concentration of some metals necessary in enzymatic processes, thereby completely altering the pathogenic process of fungi [29]. Recently, alterations in the respiratory capacity of pathogenic fungi after applying chitosan have been reported [30].

Although there are reports indicating that chitosan is effective in inhibiting the development of *Colletotrichum* sp. in various plant systems [31], the mechanisms of action are not entirely clear. To clarify at the molecular level the possible mechanisms of action of chitosan, it is important to understand

the gene expression involved in the fruit–pathogen–chitosan interaction. Currently, there is little information concerning the activity of genes related to the defense system of fruit during the interaction with chitosan and the pathogen; therefore, information concerning gene activity is of great importance to understand and define the processes involved in this interaction.

***In Vitro* Effects of Chitosan**

In vitro studies have evaluated mycelial growth, sporulation, spore germination, and hyphal and conidial morphology. The different types of fungi have differential responses in relation to the effect of chitosan, indicating that these responses are obtained by the chitosan concentrations applied, degree of polymerization, deacetylation level, and exposure time of chitosan with fungal structures [32–35]. Numerous reports mention significant reductions in post-harvest deterioration of many fruits and vegetables when treated with different doses of chitosan before storage, slowing the onset and process of infection [36].

López-Mora et al. [14] applied chitosan to the fungus *A. alternata*, isolated from fruit of mango cv. Tommy Atkins, and observed gradual control over the growth and development of the fungus as the chitosan concentration increased. Similar results were obtained in the *Colletotrichum–Fusarium*–banana interaction system, where the use of chitosan at 1.5% and 2.0% yielded 100% growth inhibition in both cases. As for spore germination, 100% inhibition was observed for both fungi using 1% chitosan [37].

***In Vivo* Effects**

Chitosan can prolong the shelf life of fruits and vegetables by forming a semi-permeable layer, which regulates gas exchange and reduces transpiration losses. This effect has been reported in many fruit and vegetable products, such as strawberries, apples, tomatoes, and red peppers, among others. In the ripening process, it has been reported that chitosan is involved in synthesis modification and ethylene and CO₂ production in apples (*Malus domestica*), cucumbers (*Cucumis sativus*), and peppers (*Capsicum annuum*) [38]. In other parameters related to fruit quality, a positive effect has also been shown by applying chitosan, as weight loss decreases, fruit firmness and titratable acidity increase, and the content of total soluble solids tends to be maintained or increased [39–40].

Results reported by Berúmen et al. [41] and Ramos-Guerrero [42] mention that applying 1% chitosan films in fruit of mango cv. Tommy Atkins and soursop (*Annona muricata*) significantly controlled the presence of *C. gloeosporioides*. Furthermore, chitosan application significantly reduced weight loss compared to untreated fruit. It was also observed that changes in firmness, total soluble solids, pH, and acidity were slower in chitosan-treated

fruit, so the postharvest quality of fruit treated with this polymer relative to firmness and physiological weight loss prolonged their shelf life by at least 5 days (stored at 20°C) compared to untreated fruit.

CHITOSAN AS A RESISTANCE INDUCER

The use of chitosan as a resistance inducer may be significant if the systemic and persistent nature of the defense proteins in plant tissues in response to the presence of chitosan, which may be important in delaying the resumption of a latent infection that typically begins to be activated when tissue resistance declines, is considered [43–46]. One of the current trends in the control of postharvest diseases is to stimulate the fruit and vegetable product to revive its own defense mechanisms [47–48].

Zhang et al. [49] indicated that as an exogenous inducer, chitosan can activate resistance in the host (fruit) by increasing the activities of various defense-related enzymes, such as chitinase and β -1,3-glucanase (GLU) in oranges (*Citrus sinensis*), strawberries (*Fragaria x ananassa*), and raspberries (*Rubus idaeus*) [50,51], and the activity of phenylalanine ammonia-lyase (PAL) in strawberries and table grapes (*Vitis vinifera*) [52–54]. In this regard, Meng et al. [55] reported similar results in pear fruit after being treated with chitosan or oligochitosan, in that they induced enzymatic activities of peroxidase (POD), polyphenol oxidase (PPO), chitinase (CHI), and GLU, which was beneficial for the fruit against infection by phytopathogenic fungi. Liu et al. [56] found that chitosan treatment induces PPO and POD activities and increases phenolic compound content in tomato fruit. Berúmen [41] reported that it was possible to induce activity of the PPO and POD enzymes, which are considered important enzymes in the defense system of plants, with the 1% chitosan concentration obtaining the best results, since it succeeded in inducing enzymatic activity of both in mango fruit.

The information that exists concerning induction by chitosan is mainly related to the analysis of enzymatic activity of proteins involved in pathogen defense in plants or in some temperate climate fruits [46,51–53,55,57–59]. In strawberry, various inducers were tested on the expression of 18 defense genes promoting the expression of genes involved in the pathway of the phenylpropanoids, polygalacturonase, and glucanase, among others [60]. Regarding the information generated in tropical fruits related to the inducing ability of chitosan, it is scarce and even more so in regard to the use of molecular analysis to evaluate the effect of chitosan at the genetic level and gene expression by real-time polymerase chain reaction (RT-PCR) of the genes involved in

a plant or fruit defense system. There are few reports that evaluate the effect of chitosan on the pathogen, at the spore or mycelial level in terms of gene expression, in fungi as important from the postharvest standpoint as the genus *Colletotrichum* [6–9,26,61,62]. Berumen [41] reported that applying 1% chitosan films in mango fruit resulted in an increase in gene expression in the early-onset stages of infection by *C. gloeosporioides* in mango fruit, suggesting that the chitosan acts from contact with the host through a signal transduction process, thus achieving induction and an increase in the gene expression of both PPO and POD, activating the defense mechanisms of the fruit [41].

Hernández-Ibañez [37] evaluated NPR1 (gene related to defense systems) gene expression with 1.5% chitosan on banana fruit inoculated with *C. gloeosporioides*. In this study, high gene expression in the early-onset stage of the pathogen–fruit–chitosan interaction was observed. Ochoa-Jiménez [62] analyzed the expression of the PG-encoding gene in spores of *C. gloeosporioides* isolated from banana fruit. A decrease was observed in the expression of the PG-encoding gene, due to the application of 1% chitosan, which resulted in an alteration in the spore germination process of this microorganism.

Currently, there is little information regarding the activity of genes related to the defense system of fruit in interaction with chitosan and the pathogen *C. gloeosporioides*. These data are important to understand and define the metabolic processes produced in fruit when its defense system has been induced. Recently the antifungal activity of low and medium molecular weight chitosan (LMWC and MMWC, respectively) was evaluated, and a transcriptomic analysis of genes with differential expression induced by chitosan in fruit of avocado (*Persea americana*) cv. Hass was performed [64]. The results showed that the chitosan concentration was directly proportional to inhibition of the pathogen, with 1.5% concentrations of LWMC and MWMC achieving significantly greater inhibition (90%) in isolated *Colletotrichum* strains.

In addition to the above, molecular evidence of the effect of chitosan was obtained by transcriptomic analysis of the fruit–pathogen–chitosan interaction. By analyzing the expression profile, genes that are repressed due to the effect of the pathogen were identified, thus providing information on the pathogenic process (fruit–plant pathogen). In turn, chitosan induces the expression of genes involved in the biosynthesis of some compounds such as dienes and certain phenolic compounds that have been reported to participate in the defense system of fruit [64]. The information obtained from this transcriptomic analysis is of great importance since it provides comprehensive information on the different metabolic pathways that may be involved both when the disease develops and when chitosan-induced resistance is

generated in fruit; thus, from the analysis of overall gene expression, arguments are obtained to clarify the mechanism(s) by which chitosan is able to induce the defense system of fruit, contributing to the development of new strategies with a molecular approach aimed at fighting diseases such as anthracnose, preventing losses in avocado crop production.

DIFFERENTIAL GENE EXPRESSION ANALYSIS

Comparing the avocado fruit that were not treated with chitosan with those that were inoculated (Figure 11.1) showed 3200 differentially expressed genes (for all times), while the fruit that were treated with chitosan (Figure 11.2) had 5075 differentially expressed genes, showing that there is

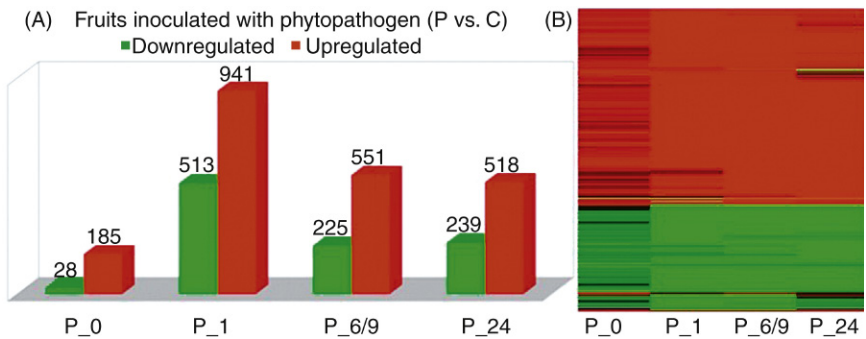


Figure 11.1 Differential Expression of Sample Inoculated with *C. gloeosporioides* (P) Compared with Control Fruits (C). (B) Expression profile of the genes present in the graph in part (A). Color black in the expression profile represents genes in basal state

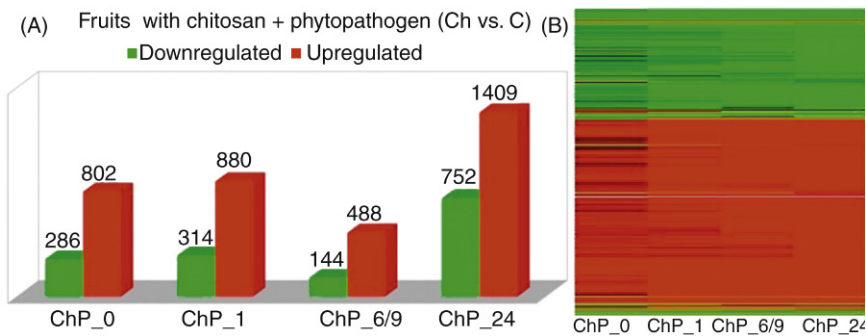


Figure 11.2 Differential Expression in Fruit after Treatment with Chitosan and Inoculated with *C. gloeosporioides* (ChP) Compared to Control (C). (B) Expression profile of the genes present in the graph in part (A). Color black, in the expression profile represents genes in basal state.

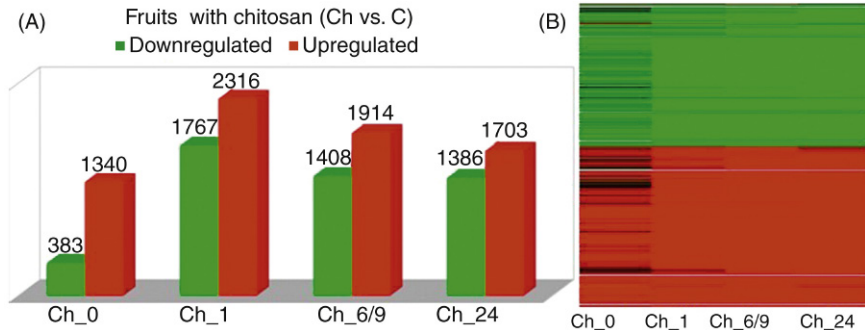


Figure 11.3 *Differential Expression in Fruit after Treatment with Chitosan (Ch), Compared to Control (C).* (B) Expression profile of the genes present in the graph in part (A). Color black, in the expression profile represents genes in basal state.

a greater differential gene expression due to the effect of chitosan–pathogen in the fruit (Figure 11.3).

Once the differentially expressed genes were determined, they were analyzed by MapMan classification [64]. In this database, genes can be placed according to their association with a specific function within the cell (Figure 11.4). The set of genes described with differential expression in response to chitosan are involved in different processes where a considerable

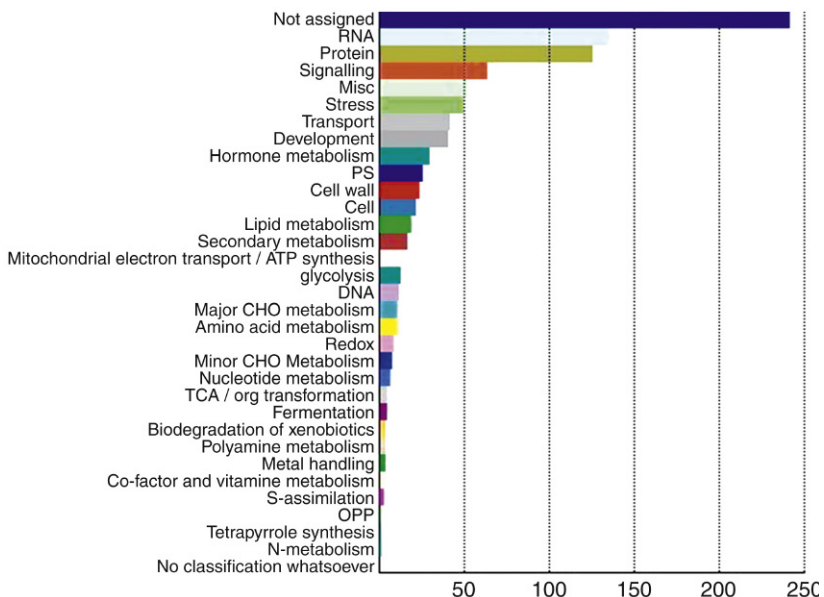


Figure 11.4 *Functional Annotation (MapMan) Differential Expression Genes in Fruit Samples after Treatment with chitosan, Compared to Control.*

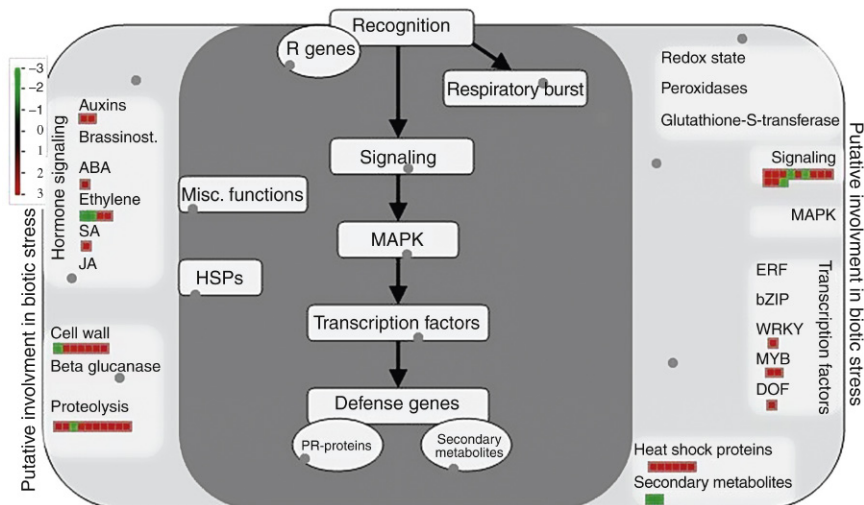


Figure 11.5 MapMan Classification of Genes Involved in the Metabolic Pathway Biotic Stress for Fruit Inoculated with *C. gloeosporioides* (P). Light-gray (green in the web version) squares representing downregulated genes. Dark-gray (red in the web version) squares representing upregulated genes.

number of genes that participate in both biotic and abiotic stress response (Figure 11.5) and some related to the metabolism of hormones such as jasmonates, salicylic acid, and ethylene (Figure 11.6), which play an important role in response to biotic stress and are also actively involved in the fruit defense system, can be highlighted [65–70].

CONCLUDING REMARKS

Chitosan has shown great potential as a natural, biodegradable substance with antifungal activities. It induces defense responses at the fruit level and directly alters the pathogen at the mycelial and spore level. However, our knowledge of the mode of action is limited. Therefore, to fully understand the mechanism used by chitosan against pathogenic fungi and its function in inducing the defense response of fruits to infection by pathogens, new approaches are required in the analysis at the molecular, transcriptomic, and proteomic level. Analyzing the differential expression of genes and proteins will provide more elements to more completely understand the mode of action of chitosan at the pathogen or plant system level, in the postharvest phase of tropical fruits, and also provide guidelines to achieve sustainable agriculture in the near future.

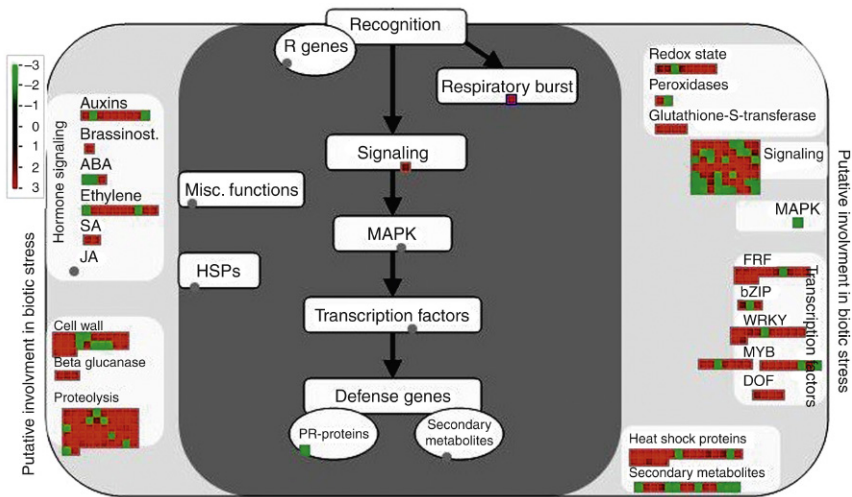


Figure 11.6 *MapMan classification of Genes Involved in the Metabolic Pathway Biotic Stress for Fruit after Treatment with Chitosan (Ch).* Light-gray (green in the web version) squares downregulated genes. Dark-gray (red in the web version) squares upregulated genes.

According to the expression profiles and functional annotation, chitosan has an impact on metabolic pathways related to the plant defense system, and it also has an effect on other pathways important in fruit development. It can also be seen that chitosan acts as an elicitor (inducer) of the defense system in fruits and is able to induce genes related to substances involved in this pathway.

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PART 4

Chitosan Bio-Nanocomposites

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CHAPTER 12

Chitosan-Based Bionanocomposites: Development and Perspectives in Food and Agricultural Applications

Cynthia Nazareth Hernández-Téllez, Maribel Plascencia-Jatomea, Mario Onofre Cortez-Rocha

INTRODUCTION

At present, there is considerable interest in processing polymeric composite materials filled with nanosized rigid particles called “nanocomposites.” In these two-phase materials, one of the phases has at least one dimension lower than 100 nm. Nanomaterials constitute an increasingly important nanometric scale product that has become significantly relevant in recent decades. Different nanoparticle (NP) systems have been developed with alternative functions in the industrial area, including food packaging, and particularly in the field of medicine, where they are considered a significant tool. Nanomaterials are used as therapies for the treatment of different diseases, as carriers for controlled drug release, and in medical engineering, among others. Nowadays the development and application of nanocomposites are at a peak. Therefore, due to competition and commercial progress, it has been necessary for companies to focus on innovation and improvement of their productivity in terms of safety, including the use of sustainable packaging and implementation of flexible and standardized technology. Recent progress in nanotechnology provides large companies with the opportunity to rearrange their processes, packaging, and manufacturing schemes [1]. This technology can be used in various areas and allows the development of a wide range of products within the food, medicine, and pharmaceutical industries. Nanomaterials not only include metallic, bimetallic and metal oxide, but also polymeric NPs, as well as advanced materials such as carbon nanotubes and dendrimers; however, considering environmental hazards,

research in this area has been focused on other biodegradable materials that form the basis of “*green nanotechnology*.”

For this reason, nanocomposites made from natural polymers with bioactive compounds are a promising area in the development of bionanotechnology. Due to their biocompatibility, biological activity, and biodegradability, they have had an important role in medicine and pharmaceuticals. Biopolymers such as starch, cellulose, poly(lactic) acid, and chitosan are of increased interest due to consumer awareness of environmental concerns, increased price of crude oil, and global warming. Renewability, low or nontoxicity, biocompatibility, and antimicrobial activity, among other factors, make chitosan-based biocomposites desirable in variety of applications, such as therapeutic aids, medicines, functional coatings, food products, and antimicrobial packaging materials.

Biocomposites are composite materials comprising one or more phase(s) derived from a biological origin. In terms of the reinforcement, they may include plant fibers such as cotton, flax, hemp, or fibers from recycled wood or waste paper, or even by-products from food crops. The manufacturing of true biocomposites demands that the matrix be made predominantly from renewable resources, although the current state of biopolymer technology dictates that synthetic thermoplastics and thermosets dominate commercial biocomposite production. In this regard, several nanoparticulated systems have been developed using biopolymers as a base, the study of which has focused primarily on the encapsulation efficiency of the active compound added to the polymer matrix [2] and loading capacity [3] in the controlled-release rates [4] and in various chemical and physical analysis [5]. However, few studies document the toxicity and biosafety of these NPs in several biological systems.

The NPs have highly particular characteristics that can fail when exposed to living organisms including humans, animals, and plants. Because of their size, they possess the ability to enter the cell membranes, having an area/volume ratio that provides them with a large contact surface and high reaction capacity. Because of these intrinsic characteristics, added to the fact of being made from natural compounds, the NPs may be toxic. This toxicity is associated with cellular damage caused by an accumulation in tissues and organs as a result of exposure to a given dose.

Agriculture is one promising area of application for chitosan-based NPs as a controlled-release system. Agriculture and the food it produces are becoming increasingly important in a world of diminishing resources and an ever-increasing global population. Given this increasing world population, it is necessary to use modern technologies such as nanotechnology and nanobiotechnology in agriculture and food sciences. Strategies to apply

nanoscience to the food industry are quite different from the more traditional applications in biosciences and engineering. Food processing is a multitechnological manufacturing industry that involves a wide variety of raw materials, high biosafety requirements, and well-regulated technological processes. Four major areas in food science and production may benefit from nanotechnology: development of new functional materials (NPs, nanoemulsions, nanocomposites, nanostructured materials), microscale and nanoscale processing (heat/mass transfer, nanoscale reaction engineering, nanobiotechnology, molecular synthesis), product development (delivery, formulation, packaging), and methods and instrumentation design for improved food safety and biosecurity (nanosensors, nanotracers) [6,7].

Nanotechnology has a tremendous potential to revolutionize agriculture. Nanoagriculture focuses currently on target farming that involves the use of nanosized particles with unique properties to boost crop and livestock productivity. Agri-food nanotechnology is multidisciplinary in nature, and application to the agriculture and food sectors is relatively recent compared with its use in drug delivery and pharmaceuticals [7,8]. These applications include agriculture, hydroponics, agrochemicals, livestock, nanobiotechnology, nanotoxicology, nanotechnology, nanofood, functional nanocomposites, and biotechnology. Nanotechnology has the potential to protect and monitor plant growth, detect plant and animal diseases, increase global food production, enhance food quality, and reduce waste for sustainable intensification [7]. Food and agricultural production are among the most important fields of nanotechnology application.

Nanotechnology in agriculture has gained momentum in the last decade with an abundance of public funding, but the pace of development is modest, even though many disciplines come under the umbrella of agriculture. This could be attributed to (i) the scale of demand of input materials always being gigantic in contrast with industrial nanoproducts; (ii) an absence of control over the input nanomaterials in contrast with industrial nanoproducts and because their fate has to be conceived on the geosphere; (iii) the time lag of emerging technologies reaching the farmers' fields, especially given that many emerging economies are unwilling to invest in innovation; and (iv) the lack of foresight resulting from agricultural education, where personnel from kindred disciplines might lack an understanding of agricultural production systems. If these issues are addressed, nanotechnological intervention in farming has bright prospects for improving the efficiency of nutrient use through nanoformulations of fertilizers, breaking yield barriers through bionanotechnology, surveillance and control of pests

and diseases, understanding the mechanisms of host-parasite interactions at the molecular level, development of new-generation pesticides, preservation and packaging of food and food additives, removal of contaminants from soil and water, and improving the shelf life of vegetables and flowers, to name a few [9].

In this review, issues related to the development of bionanocompounds prepared from chitosan and bioactive compounds are discussed, as well as the toxic and antimicrobial effect of both chitosan and chitosan bionanocomposites. Aspects related to the main NP's obtaining methods and environmental implications associated with the use of nanomaterials in the agricultural area are also considered.

CHITOSAN

Source and Chemical Properties

The term *chitosan* describes a heterogeneous group of polymers that differ in molecular weight (MW), viscosity, degree of deacetylation, and pKa, among other physiochemical characteristics. Chemically it is a binary linear high MW copolymer comprised of β -1,4-D-glucosamine and N-acetyl-D-glucosamine chains. It constitutes the only cationic polysaccharide from natural origin. The physical properties of the polymer vary according to a number of parameters such as MW (of approximately 10,000–1,000,000 Da), deacetylation degree (ranging between 60% and 95%), and the purity and sequence between the amino and acetamide groups. Due to its specific and rigid structure, chitosan is able to exist in nature in different polymorphic forms, whose properties vary considerably [10].

Chitosan is obtained by partial chemical or enzymatic deacetylation of chitin, which is the second most abundant natural polymer after cellulose, with a regeneration rate of 10^9 – 10^{11} tons per year in the biosphere. The main source of chitinous polymers is crustacean shells, cuticles from insects, and cell wall of fungi. The amino group of chitosan allows the polymer to be soluble in diluted organic acids and the degree of deacetylation determines the number of amino groups present in the polysaccharide, thus influencing their functionality [11].

Chitosan is considered to be a biologically compatible polymer for humans and most animals, and is approved for use in diet in Japan, Italy, and Finland. The chitosan obtained from shrimp was designated as GRAS (generally recognized as safe) on 2005 by the USFDA (US Food and Drugs Administration), based in the scientific procedures for use in food in general, in

accordance with Good Manufacturing Practice regulations [10]. However, some modifications implemented in chitosan can generate chitosan derivatives with a degree of toxicity, and like any residual reactive, it should be handled with care.

In addition to biocompatibility and low or null toxicity, chitosan has interesting properties, such as biodegradability and versatile physicochemical properties, which make it attractive; it has had a wide range of applications in several fields such as food, cosmetics, biomedicine, agriculture, and wastewater management [1,8,10]. A novel application is in the design of functional and biodegradable materials, which constitutes a promising alternative to the use of polymeric materials that result from the constant production of these leaders in environmental pollution [12]. One of the applications of interest for chitosan is the development of biodegradable materials, with potential application both in food packaging for preservation and shelf-life extension [13], and in agriculture for the release of bioactive compounds, an understudied area of application.

Toxicity

Current studies show that, in general, chitosan is a relatively nontoxic and biocompatible material (Table 12.1). However, care must be taken to ensure that it is pure, as protein, metal, or other contaminants could potentially cause many deleterious effects both in derivative syntheses and in dosage forms. After derivatization (or cross-linking), unreacted reagents should be thoroughly removed to prevent confounding results as many reagents are cytotoxic uncoupled. Many enzymes seem to have activity, at least *in vitro*, against chitosan but derivatives could give indigestible molecules. The biodistribution is both MW and formulation dependent, with relatively long circulation times achievable. The biodistribution is controllable through route of administration, dosage form, and chitosan characteristics. Intravenous distribution can be tuned using particle size (<100 nm), and the liver appears to be a primary site of localization [14].

It has been reported that chitosan derivatives do not generate any chronic toxicity in *in vivo* animal models. No death or other signs of toxicity are observed in mice during the peroral treatment with different derivatives of chitosan (30 mg/kg body weight for 1 month), thus establishing their safe application in oral insulin delivery. Also, no changes are observed in the architecture of the internal organs such as liver and kidney of the treated groups. Moreover, studies have shown that the intestinal luminal microbes cause successful biodegradation of these polymers (chitosan and their derivatives) [15].

Table 12.1 Toxicity of chitosan and chitosan derivatives

Chitosan details (DD, MW)	Modification	Assessment	IC ₅₀
95% DD, 18.7 kDa	Steric acid conjugation micelle	<i>In vitro</i> , A549 cells	369 ± 27 µg/mL
95% DD, 18.7 kDa	Steric acid conjugation and entrapment in micelle	<i>In vitro</i> , A549 cells	234 ± 9 µg/mL
97% DD, 65 kDa	<i>N</i> -octyl-O-sulfate	<i>In vitro</i> , primary rat hepatocytes	>200 mg/mL
87% DD, 20, 45, 200, 460 kDa	None, aspartic acid salt	<i>In vitro</i> , Caco-2 cells, pH 6.2	0.67 ± 0.24, 0.61 ± 0.10, 0.65 ± 0.20, 0.75 ± 0.16 mg/mL
87% DD, 20, 45, 200, 460 kDa	None, glutamic acid salt		0.56 ± 0.10, 0.48 ± 0.07, 0.35 ± 0.06, 0.46 ± 0.06 mg/mL
87% DD, 20, 45, 200, 460 kDa	None, lactic acid salt		0.38 ± 0.13, 0.31 ± 0.06, 0.34 ± 0.04, 0.37 ± 0.08 mg/mL
87% DD, 20, 45, 200, 460 kDa	None, hydrochloride salt		0.23 ± 0.13, 0.22 ± 0.06, 0.27 ± 0.08, 0.23 ± 0.08 mg/mL
78% DD, <50 kDa	None, lactic acid salt	<i>In vitro</i> , B16F10 cells	250 mg/mL
82% DD, 150–170 kDa	None, lactic acid salt	<i>In vitro</i> , B16F10 cells	2.00 ± 0.18 mg/mL
>80% DD, 60–90 kDa	None, glutamic acid salt	<i>In vitro</i> , B16F10 cells	2.47 ± 0.14 mg/mL
77% DD, 180–230 kDa	None, lactic acid salt	<i>In vitro</i> , B16F10 cells	1.73 ± 1.39 mg/mL
85% DD, 60–90 kDa	None, hydrochloric acid salt	<i>In vitro</i> , B16F10 cells	2.24 ± 0.16 mg/mL
81% DD, 100–130 kDa	None, hydrochloric acid salt	<i>In vitro</i> , B16F10 cells	0.21 ± 0.04 mg/mL
100% DD, 152 kDa	Glycol chitosan	<i>In vitro</i> , B16F10 cells	2.47 ± 0.15 mg/mL
100% DD, 3–6 kDa	20, 44, 55% Trimethyl chitosan, chloride salt	<i>In vitro</i> , MCF7 and COS7 cells, 6 h & 24 h	>10 mg/mL

100% DD, 3–6 kDa	94% Trimethyl chitosan, chloride salt	<i>In vitro</i> , MCF7, 6 h	1.402 ± 0.210 mg/mL
100% DD, 3–6 kDa	94% Trimethyl chitosan, chloride salt	<i>In vitro</i> , COS7, 6 h	2.207 ± 0.381 mg/mL
100% DD, 100 kDa	36% Trimethyl chitosan, chloride salt	<i>In vitro</i> , MCF7, 6 h	0.823 ± 0.324 mg/mL
100% DD, 100 kDa	36% Trimethyl chitosan, chloride salt	<i>In vitro</i> , COS7, 6 h	>10 mg/mL
84.7% DD, 400, 100, 50, 25, 5 kDa	40% Trimethyl chitosan	<i>In vitro</i> , L929 cells, 3 h	30, 70, 90, 270, >1000 µg/mL
84.7% DD, 1.89 MDa	12% PEG modified 40% trimethyl chitosan	<i>In vitro</i> , L929 cells, 3 h	220 µg/mL
84.7% DD, 3.6 MDa	25.7% PEG modified 40% trimethyl chitosan	<i>In vitro</i> , L929 cells, 3 h	370 µg/mL
84.7% DD, 300 kDa	6.44% PEG modified 40% trimethyl chitosan (and all PEG modified TMC with lower Mw)	<i>In vitro</i> , L929 cells, 3 h	>500 µg/mL
97% DD, 65 kDa	<i>N</i> -octyl-O-sulfate	<i>In vivo</i> , 1V, mice	102.50 mg/mL
97% DD, 65 kDa	<i>N</i> -octyl-O-sulfate	<i>In vivo</i> , 1P, mice	130.53 mg/mL

Source: Kean and Thanou [14].

Chitosan can be found in many shapes and sizes, varying in their degree of deacetylation (DD) and average MW, which have impacts on their biological activity and toxicity. In a controlled-release system, chitosan normally act as carrier excipient of the active compound, therefore its internalization, distribution, and toxicity should be studied following its consumption.

It is considered that in vertebrates, chitosan is degraded in the colon and different enzymes act, mainly lysozyme and bacterial enzymes. Nevertheless, three different human chitinase enzymes showing activity have been identified. In general, microorganisms synthesize and/or degrade chitin, the natural precursor of chitosan, through the use of microbial chitinases that hydrolyze the *N*-acetyl- β -1,4-glucosamine joints randomly. The rate of biodegradability in living organisms depends on the chitosan deacetylation degree; if the deacetylation degree increases, the degradation rate decreases [14,16].

Antimicrobial Activity

Chitosan has antimicrobial activity against certain microorganisms such as filamentous fungi, yeast, and Gram-positive and Gram-negative bacteria. This activity is more bacteriostatic than bactericidal and depends on the type and concentration of chitosan, its MW and degree of deacetylation, the type of microorganism, and microbial growth time, among other factors. However, the degree of deacetylation is the factor having the most influence on the antimicrobial activity of chitosan, because the amount of free amino groups in the molecule has been related to this activity. The antimicrobial activity is different among the strains, which implies a more complex antimicrobial mechanism than anticipated [8,11].

Several mechanisms have been proposed to explain the antimicrobial activity of chitosan, and the main mechanism is the charge interaction, which is attributed to the interaction between the positive charges of chitosan and the negative charges of cell walls. Another mechanism involves the chelating activity of chitosan amino groups on trace metals outside cells (Fe, Cu, Zn, and Mo), which are essential for the growth of an organism. The interference in protein synthesis as a consequence of the membrane damage caused by charge interaction with chitosan is a mechanism that involves the ability of chitosan to pass through the cell membrane of a microorganism, and subsequently bind to DNA and interfere with protein synthesis. Experimental results show that charge interaction as an antimicrobial mechanism of chitosan involves the following: cell permeability alteration, cell membrane structural damage, and charged group interactions [8,17].

The ability of chitosan to pass through cell membranes apparently depends on the membrane composition, those cells with a high content of polyunsaturated fatty acids being more sensitive to chitosan. Palma-Guerrero et al. [18] observed a greater antifungal effect of rhodamine-labeled chitosan on *Fusarium oxysporum* than on *Pochonia chlamydosporia*. Chitosan penetrated cell membranes of *F. oxysporum* by plasma membrane permeabilization, resulting in cell lysis.

When used to enhance plant defenses, chitosan induce host defense responses in both monocotyledons and dicotyledons. To counteract the negative effect or contamination caused by viruses, fungi, bacteria, and insects, plant cells treated with chitosan increase their production of phytoalexins, protease inhibitors, lignin, oxygen-reactive species, phenolic compounds (ferulic acid, salicylic acid, *p*-Coumaric acid), and defense enzymes such as chitinases, chitosanases, glucanases, and phenylalanine ammonia-lyases. According to the induction mechanism in vegetal cells, chitosan activates the production of 1,3- β -glucan synthase in the plant cell wall, resulting in (i) the accumulation of β -glucan, (ii) changes in protein phosphorylation/dephosphorylation reactions, (iii) an increase in internal calcium concentration, (iv) a decrease in internal potassium concentration, (v) a decrease in internal pH, and (vi) changes in jasmonic acid production [8]. Responses also include ion flux variations, cytoplasmic acidification, membrane depolarization and protein phosphorylation, and the expression of unique early responsive and defense-related genes. In addition, chitosan was reported to induce callose formation, proteinase inhibitors, and phytoalexin biosynthesis in many dicot species. The response to chitosan and derived oligosaccharides varies with their acetylation degree [19]. The structural shape and physical condition found in chitosan is a crucial factor and has great influence in its antimicrobial activity; this aspect should be highly considered, yet is often underestimated [20].

In agriculture, some fungi produce polluting toxins during the storage of various products, causing significant economic losses associated with impacts on human health, animal productivity, and international trade, which has attracted the attention of scientists and politicians worldwide. It has been estimated that 25% or more of agricultural products are contaminated with mycotoxins. The aflatoxins are termed as polyacetic natural compounds and are produced mainly by *Aspergillus flavus* and *Aspergillus parasiticus* at the end of the exponential growth phase; they are the main pollutants of products such as corn, peanut, cotton, nuts, rice, tobacco, and spices [21].

Aflatoxin B1 is the most important due to its high toxicity to humans and animals. Depending on the duration and level of exposure, aflatoxins are hepatotoxic and carcinogenic. Cota-Arriola et al. [22] evaluated the fungistatic activity of chitosan against *A. parasiticus*, finding an inhibitory effect to concentrations of 6.7 and 10.7 g/L (CI_{50}) to the biopolymer, which made it possible to slow the growth of the fungus by 50% at 122 h. Thus, the chitosan (CI_{50}) strongly inhibited the radial growth until 122 h and the germination of spores by more than 50% before 8 h. Compared with the control, the chitosan significantly decreased Aflatoxin B1 production at 8 d; however, the total *A. parasiticus* aflatoxins production increased notably.

The use of chitosan for the control of postharvest diseases caused by toxigenic fungi has attracted considerable attention because of the problems associated with the use of chemical agents, which also negatively affect environmental pollution through the generation of hazardous waste in food products and the growth of chemical treatment-resistant fungi species. This has increased interest in the study and use of antimicrobial compounds obtained from natural sources. Antimicrobial properties of chitosan, which have been widely documented, constitute a starting point for the study and development of nanoparticulated systems elaborated with natural bioactive compounds, designed to counteract different problems that can be resolved through the use of this technology [8,23]. In this regard, there are different studies on the antimicrobial activity of chitosan NP systems, which are commonly accompanied by an active compound for their release at a certain system. This active compound can have an organic or inorganic origin. Recent studies have demonstrated the metallic NPs have a strong antimicrobial effect; however, it is also stipulated that prolonged exposure to this type of nanomaterials can lead to toxicity [24].

Chitosan-Based Bionanocomposites

NP systems represent an emerging group of hybrid materials that are formed by a combination of natural polymers and inorganic solids and exhibit a structural improvement and functional properties of great interest for different applications. The inherent properties of biopolymers, such as its compatibility and biodegradability, open new perspectives associated with the development of bionanocomposites, with special applications in agriculture and the development of environmentally friendly materials [13].

It has been suggested that the inherent properties of packaging materials based on biopolymers can be surpassed by technology of nanocomposites, which exhibit increased barrier properties, increased mechanical strength,

and improved resistance compared to the pure polymers and conventional compounds [25].

Bionanocomposites or “green nanocomposites” open an opportunity for the use of novel functional materials based on their high yield that can replace conventional oil-based plastic packaging materials [26]. Thus the development of biologically based polymers such as chitosan and the use of innovative technologies can reduce dependence on fossil fuels. However, the new physicochemical properties of these nanomaterials emphasize the need to adequately evaluate the potential effects and risks to human health, specifically regarding possible cytotoxicity. Even though evidence suggests that these materials can have an adverse effect on human health, the cause/effect relationship is not yet fully understood [27].

Over the past era, research in functional biomaterials such as chitosan has led to the development of new drug delivery systems and superior regenerative medicine, currently one of the most quickly growing fields in the area of health science. Chitosan, well known due to its biological properties and versatility, clearly has greater potential for future development in different fields of science, namely drug delivery, gene delivery, cell imaging, and sensors; in the treatment as well as diagnosis of some diseases such as cancer; and recently in food and agriculture. Chitosan-based nanomaterials have superior physical and chemical properties such as high surface area, porosity, tensile strength, conductivity, and photoluminescence as well as increased mechanical properties in comparison to pure chitosan [28].

At present, a large number of studies have focused on antimicrobial or pesticide encapsulates, establishing the dosage required to control microbial growth; however, this method of control does not completely solve the problems associated with the indiscriminate use of synthetic chemicals. For this reason, it is necessary to continue the search for viable natural alternatives capable of forming controlled-release matrices for agricultural use, such as chitosan, which may hold active compounds with antimicrobial activity [8].

Many studies examine the encapsulation of compounds in chitosan-based micro- and NPs. Because the pharmaceutical industry is the main area of application of chitosan-based matrices for the controlled release of drugs, most of the processes to obtain micro- and NPs have been adapted to this area [29]. Currently, human implant materials based on chitosan have been designed in the biomedical area; they exhibit high antibacterial activity that can be used to control potential infections. These materials are based

mainly on the use of micro- and NPs of chitosan with metal ion incorporation to increase their antibacterial potential [30]. The study of controlled-release matrices based on chitosan has been focused on pharmaceutical and biomedical sciences; therefore, it is important to apply this technology to other areas of research such as food and agriculture that have not been well explored. Table 12.2 shows some studies and applications of compounds encapsulated in chitosan-based matrices [8].

Plagues are among the main causes of agricultural losses; therefore, insecticides are an important control tool. However, collateral effects such as environmental contamination, human poisoning, reduction in the number of natural enemies, and insecticide resistance by plague insects, among others, may be credited to their indiscriminate use. Formulations containing pesticides have been prepared in colloidal suspensions or powder in nanoscale, where they present advantages such as increasing stability of the active organic compound (UV, thermal, hydrolysis, etc.), foliar settling, reduction in foliar leaching, systemic action, synergism, and specificity. Consequently, the amount of pesticide necessary, the number of applications, and the environmental impact, along with human exposure to pesticides are reduced. The nanoformulations have been employed not only for synthetic pesticides but also in alternative natural products (herbal extracts) and entomopathogenic microorganisms to control insect pests [31].

METHODS FOR OBTAINING NANOPARTICULATED SYSTEMS

Controlled-release nanoparticulated systems vary in their structure and morphology. They can be particulate, in the form of fibers, capsules, layers, or micelles, all of them in a nanometric scale. The final shape and functionality depends on the elaboration method. In this regard, several methods have been developed for preparing and obtaining chitosan-based microparticles and NPs. Emulsion-drip precipitation, cross-linking, spray drying, ionic gelation, and nanoprecipitation are among the most commonly used methods.

Emulsion-Droplet Coalescence Method

The emulsion-droplet coalescence method developed by Tokumitsu et al. [32] utilizes the principles of the emulsion, both cross-linking and precipitation. However, in this method, instead of stable drops cross-linking, precipitation is induced by allowing chitosan droplet coalescence with NaOH drops. First, a stable emulsion containing an aqueous chitosan solution along

Table 12.2 Food and agricultural applications of active compounds encapsulated in chitosan-based controlled-release matrices

Matrix	Bioactive compound	Characteristics
Food applications		
Chitosan and chitosan/alginate microparticles	Tannic acid (antioxidant)	Diameter of 3–6 μm , entrapment efficiency of 14–28.9%
Chitosan/glutaraldehyde microcapsules (emulsion/casting)	Astaxanthin (antioxidant)	Spherical shape, diameter of 5–50 μm
Chitosan NPs (ionic gelation cross-linked sodium tripolyphosphate)	Catechins (antioxidant)	Encapsulation efficiency of 24–53%
Chitosan microspheres (spray drying)	Olive leaf extract (antioxidant)	Spherical shape, uniform particle size distribution, encapsulation efficiency of up to 27%
Chitosan microspheres (ionic gelation cross-linked sodium tripolyphosphate)	Vitamin C (antioxidant)	Size of 3.1, 4.9, and 6.7 μm , encapsulation efficiency of 67.25, 60.43, and 52.74%
Chitosan microcapsules (O/W emulsification)	Volatile citronella oil (flavor)	Size of 225 ± 2 to 131 ± 20 μm , encapsulation efficiency of 98.2–95.8%
Chitosan/arabic gum microcapsules stabilized with sodium dodecyl sulfate	Citral and limonene (flavor)	Citral particle diameter of 0.25, 0.41, and 1.1 μm ; limonene particle diameter of 0.25, 0.37, and 0.99 μm
Chitosan NPs (ionic gelation cross-linked sodium tripolyphosphate)	Riboflavin/ β -lactoglobulin (vitamin)	Size of 100–200 nm, surface charge of 20–60 mV, encapsulation efficiency of 60%
Chitosan/linoleic acid nanocapsules (O/W emulsification with methylene chloride)	Linoleic acid (oil)	Size of 200–600 nm, encapsulation efficiency up to 50%
Agricultural applications		
Chitosan gel beads (with acetic or propionic anhydride)	Herbicide atrazine and fertilizer urea (agrochemical)	Extended release period of atrazine to 7 months; chitosan-coated urea beds extended action of urea release (up to 180 h)
Chitosan microcapsules (precipitation)	3-hydroxy-5-methylisoxazole (herbicide)	Size of 5 μm ; sustained release of active material in water for 80–160 h

Source: Adapted from Cota-Arriola et al. [8].

with the active compound is produced in liquid paraffin oil, and then another stable emulsion containing a NaOH aqueous solution is produced in the same manner. When both emulsions are mixed at high-speed agitation, the drops from each emulsion can collide randomly and merge, precipitating chitosan droplets to give small-sized particles.

Cross-Linking

This method involves the cross-linking of chitosan amino groups with aldehyde groups of a cross-linker agent. An emulsion of water in oil is prepared and stabilized with a surfactant. Then, the emulsion is combined with a cross-linking agent such as glutaraldehyde to form spheres. Subsequently, spheres are filtered and washed with hexane or dry alcohol. The particle size is controlled by the size of the emulsion dripping interacting with the cross-linking means, the cross-linking degree, and the stirring speed. The method has its inconveniences; it is tedious and may generate chemical reactions with the active agent. The parameters affecting the preparation and performance of the spheres are the MW and the concentration of chitosan, as well as the type and concentration of surfactant [33].

Spray Drying

Spray drying is a known technique to produce powders, granules, or agglomerates of mixed drug solutions, excipients, and suspensions. It is based on the drying of atomized drops in a hot air stream. In this method, chitosan is first dissolved in acetic acid in aqueous solution; the drug is dissolved or dispersed in the solution, and then the adequate cross-linking agent is added. This solution or dispersion is atomized in a hot air stream, which leads to the formation of small drops of solvent, from which it instantly evaporates and leads to the formation of freely flowing particles. Various process parameters must be controlled in order to obtain the desired particle size, which depends on the nozzle size, spray flow speed, atomization pressure, inlet air temperature, and the extent of cross-linking [33].

Ionic Gelation

The use of complexes between macromolecules of opposite charge to prepare chitosan microspheres has attracted much attention because the process is simple and smooth. Moreover, electrostatic interactions have been applied by reversible physical cross-linking instead of chemical cross-linking, to avoid the possible toxicity of the reactants and other undesirable effects. Tripolyphosphate (TPP) is a polyanion that interacts with the chitosan by electrostatic

forces. In the ionic gelation method, chitosan is dissolved in aqueous acid solution to obtain the polycationic chitosan. Then, this solution is added dropwise to the solution under constant TPP polyanionic agitation. Due to the complexity between oppositely charged species, the chitosan undergoes ionic gelation and precipitation to form spherical particles. However, the microparticles formed by TPP/chitosan have poor mechanical strength, which limits their use in the release of drugs or active compounds [23].

Nanoprecipitation

This method has numerous advantages: it is a simple, quick, and easy-to-perform technique. The formation of NPs is instantaneous and the procedure is performed in one step. It requires using two miscible solvents, the first to dissolve both the polymer used as the drug (solvent), while the latter must be insoluble for both (nonsolvent). Nanoprecipitation occurs when the polymer in solution (solvent) comes in contact with the nonsolvent; a displacement of solvent occurs and the polymer chains start to fold, leaving the active compound trapped. The predominant effect in this process is called “Marangoni,” which is the mass transfer between two turbulent phases caused, in this case, by the solvent and nonsolvent. Some of the complex phenomena involved in this precipitation system are the flow, diffusion, and variations in surface tension. Besides the chitosan, other types of polymers have also been used for the formation of NP systems through this technique, such as polylactic acid and cellulose derivatives [23,33–35].

Selecting the most appropriate method depends on the final product and the particular objectives required as well as on the particle size, thermal stability, time of release of the active compound, and the toxicity of the end products, among others. The release of the bioactive compound from particle systems will depend on the density of the polymer matrix [8,23].

TOXICITY OF CHITOSAN-BASED NPs

Despite the potential benefits that nanotechnology offers in the agri-food sector, little is known about safety aspects of the application of nanotechnologies in food production and the incorporation of NPs in food. Risk assessment procedures are not specific to agri-food nanomaterials, resulting in uncertainty regarding the nature and extent of potential risks in most cases. Applications for nanomaterials currently used for meat, and food generally, include the use of NPs and nanomaterials as food ingredients or additives that are placed directly into food or as a part of food packaging. Also, the

release of engineered NPs may cause adverse effects on edible plants, and the potential risks and benefits of using nanosilver as an antibacterial agent in consumer and health care products are being debated globally [36].

In 2012, a new biocidal product regulation (EU 528/2012) was adopted in the European Union. The regulation specifically requires assessment and approval of active nanomaterial biocidal ingredients. Also, on February 12, 2014, the European Parliament Committee on the Environment, Public Health and Food Safety rejected a proposed regulation that would define “engineered nanomaterials” in food. Such definition could lead to existing nanomaterials not being labeled due to an exemption provided for food additives approved on a European Union list [7].

As it occurs with nanostructured materials, chitosan-based elaborated NPs have a chemical composition, size, shape, solubility, electric charge, and surface structure that allow them to easily cross through membranes or link to organic compounds such as proteins. Because of this, the NPs are superior in their toxic or pharmacological action compared to other size particles, such as microparticles, even made of the same molecules. The biokinetics of these materials adjust the dose to know exactly the amount of material that reaches the organs. A dose of a drug encapsulated in NPs can lead to the onset of more severe side effects than the same dose in a common formulation [37].

With respect to its toxicity, it has been reported that cell death by apoptosis and inflammation are the most common and most widely toxic effects reported. Cell death is caused by prolonged exposure dose due to the accumulation of metals or other drugs, either within the cell or by macrophage phagocytosis [37]. Some authors have suggested possible cytotoxic effects associated with the type of salt and polymer MW [38–40]. A specific property of the NPs is their ability to cross through biological barriers by a process called translocation. This process can be found in other parts of the organism, at sites other than the entrance point, maintaining their integrity as particles without any NP dissolution occurring. Multiple factors are involved in cellular internalization routes and targeting of the main modes of uptake, endocytosis, and direct crossing of the plasmatic membrane (translocation) [37,41].

By increasing the specific contact surface of the NPs, its reactive or catalytic potential increases, which in some cases can lead to the formation of oxygen reactive species (ORS) in mammal or microbial cells [38–40]. From this it follows that the smaller a particle is, the greater its reactivity will be, so that a substance, which is inert at a micro or macro scale, can be toxic at the

nanoscale. NPs may be located within the cell in organelles where macroscopic particles fail to reach, showing specific cellular responses [8,23]. NPs of a different nature have been used in the other studies, being the metallic NPs of silver and gold, the most studied [24,37]. Despite the good results in the use of metallic NPs, several authors recommend limited use and short time exposures to these nanomaterials, because prolonged exposure may cause adverse bioaccumulation and damage to the body.

Because chitosan NPs at a size of 200 nm caused malformations (bent spine, pericardial edema, and an opaque yolk), an increased rate of cell death, high expression of ROS, and overexpression of heat shock protein 70 in zebrafish embryos [42], it is important to study the biosecurity associated with the use of chitosan NPs in agriculture. Toxicity of biodegradable nanocarriers such as chitosan NPs must be addressed, especially considering the *in vivo* distribution of these nanoscaled particles, which can involve beneficial microorganisms and animals. Despite the existing database on NPs, no blanket statements about human toxicity can be given at this time. In addition, limited ecotoxicological data for nanomaterials preclude a systematic assessment of the impact of NPs on ecosystems [43].

ANTIMICROBIAL ACTIVITY OF CHITOSAN-BASED NPs

One of the properties exhibited by the biopolymer NPs is their antimicrobial activity. Antimicrobial capacity is credited naturally to the chitosan nanoparticulated systems because it is a cationic biopolymer, being the main action mechanism destabilizing the microbial membranes with negative charges present while interacting with chitosan positive charges (—NH_2).

The size of NPs, especially high MW chitosan NPs, is greatly influenced by the concentration of chitosan that was added into a constant amount of TPP. A linear relationship was observed in which an increase in concentration would increase particle size. A similar relationship was observed with the MW of chitosan in which the effect on particle size was also prominent. The linear relationship between chitosan MW and particle size/zeta potential has been statistically proven, which provides a platform for easy manipulation of physicochemical properties of NPs suitable for their intended application. Formulation of chitosan into NPs was found to increase its antifungal effect significantly [44]. Therefore, it is anticipated that chitosan NPs have the potential of becoming a powerful and safe natural antifungal agent.

Zeta potential has been suggested as a key factor contributing to antifungal effect of chitosan through the interaction with negatively charged

microbial surface [45]. Zeta potential of chitosan NPs showed a net positive surface charge due to excess positive charge of chitosan molecules after interaction with TPP, which proved that the magnitude of particle positive charge increased linearly with the increasing concentration or MW [44].

Hsu et al. [24] found that the chitosan NPs with silver showed an antibacterial effect against *Staphylococcus aureus* at a concentration of 120 ppm. Furthermore, it was observed that small amounts of Ag NPs can induce significant changes in the physicochemical properties of chitosan, such as a good dispersion and homogeneity in the produced matrix and increased nanocrystalline domains, therefore it may increase its biocompatibility and potential in wound treatment. Silver NPs also exhibit strong antibacterial activity in addition to anti-inflammatory properties. The postulated mechanism is associated with the release of silver ions of the NPs in the presence of water that, at a nanoscale, can penetrate the bacterial wall and easily damage the structure, altering its permeability [46]. Few studies have been conducted on the antifungal effect of chitosan. One of them was carried out by Cota-Arriola et al. [23] in which they studied chitosan TPP NPs with sizes between 80 nm and 20 nm. The particles were evaluated for their antifungal potential against *Aspergillus parasiticus*, and an increased antifungal potential was observed compared to the chitosan solution. Researchers also observed damage characterized by morphological changes in hyphae and spores. NP size and the availability of the functional groups CS/TPP (amino and phosphate group) suggest a possible synergistic effect between the CS and TPP.

Nanosized chitosan NPs contribute to a larger surface area, causing them to be able to adsorb more tightly onto the surface of fungal cells and disrupt the membrane integrity. Ma and Lim [47] reported that cellular uptake of chitosan NPs into cells was higher than that of chitosan molecules because the bulk chitosan molecules were located extracellularly. This suggested that chitosan NPs might be able to diffuse into the fungal cell and hence disrupt the synthesis of DNA as well as RNA, which could explain a better antifungal activity of chitosan NPs compared to its free polymer or solution form. It has been documented that the shape, size, and nature in which a material is supplied to an organism is crucial for disposal. It is therefore important to determine whether the chitosan NPs may present a risk and bioaccumulation in living organisms.

Antimicrobial properties are attributed to chitosan NPs [8,17]. However, Leitgeb [48] found that chitosan NPs covered with magamita ($\gamma\text{-Fe}_2\text{O}_3$), at concentrations of 10–30 mg, did not exhibit inhibitory effect over the *in vitro* growth of some bacteria such as *Escherichia coli*, *S. aureus*, *Pseudomonas*

aeruginosa, *Enterococcus faecalis*, and *Klebsiella pneumoniae*. The authors concluded that the chitosan NPs do not have a toxic effect on bacteria at the concentration used, suggesting that chitosan micro- and NPs are good carriers of biologically active substances, in addition to being biocompatible and nontoxic [48].

On the other hand, native intracellular delivery of active proteins is challenging because of the fragility of most proteins. Recently, a study of chitosan NPs loaded with lysozyme as a model drug of an antimicrobial protein was performed. NPs with a diameter of 150 nm and prepared by the method of ionic gelation, effectively maintained the antibacterial activity of the enzyme (lysozyme), which was slowly released for 3 weeks *in vitro* and was active toward *Staphylococcus epidermidis* until 5 days of incubation. Furthermore, NPs loaded with lysozyme showed complete *in vitro* cytocompatibility toward murine fibroblasts [3].

CHITOSAN-BASED NANOPARTICULATED SYSTEMS LOADED WITH BIOACTIVE COMPOUNDS

In recent years the interest in developing matrices from natural polymers has increased, which results in a controlled-release system that provides a continuous dosing of functional compounds. However, in the food industry, developing versatility and highly responsive delivery systems remains a challenge [49]. The nanolayer coatings have promising applications in a wide range of areas including pharmaceutical, biomedical, and food packaging. The release of bioactive compounds of polymeric matrices can occur due to diffusion mechanism, swelling of the polymer matrix, polymer erosion, and degradation [8,49]. This behavior can prevail through different mechanisms, depending on the system and environmental conditions.

In this regard, some studies related to the release of functional compounds have been made. Pinheiro et al. [49] formulated nanolayer coatings composed of K-carrageenan and chitosan, an anionic and cationic polysaccharide respectively. This matrix was used for the release of the model cationic compound methylene blue, which was incorporated in different positions of the nanoshell. The result was that the release and transport of dye are associated with the concentration gradient and the relaxation of nanolayers, and are dependent on the temperature, pH of the medium, and the position of the methylene blue molecule incorporated into the nanolayers of K-carrageenan-chitosan, which suggests different mechanisms of action.

Xu and Du [50], using ionotropic gelation with TPP, developed chitosan NPs and assessed the release of bovine serum albumin (BSA), and found that the MW and degree of deacetylation of chitosan, the initial concentration of BSA, and the presence of polyethylene glycol (PEG) in the encapsulation medium were the factors that affect the release properties of BSA. When increasing the MW of the chitosan of 10–210 KDa, the encapsulation efficiency was doubled; furthermore, the release of BSA in PBS medium (pH 7.4) was reduced from 73.9% to 17.6% after 8 days. The increase in the degree of deacetylation from 75% to 92% slightly promoted encapsulation efficiency and slowed the release rate. Moreover, the encapsulation efficiency was significantly decreased due to the increase of the initial concentration of BSA and chitosan. A high capacity of BSA accelerated the release of the BSA of the NPs, while adding PEG challenged the encapsulation of BSA and accelerated the release rate. The authors concluded that molecular and chitosan NP formation parameters play an important role in the release of the protein, while making it possible to modulate relative parameters in order to satisfy the protein delivery needs [50].

Deng et al. [2] used chitosan NPs loaded with lysozyme prepared by ionotropic gelation, obtaining NPs that were 50–280 nm in diameter. This work focused on the MW of chitosan, encapsulation efficiency, loading capacity, rate of release, and lysozyme activity in the chitosan NPs.

Tea polyphenol-loaded chitosan NPs showed a branch shape and heterogeneous distribution in prepared suspension, and transmission electron microscope images showed in which the HepG2 cells treated with these NPs exhibited some typical apoptotic features, such as microvilli disappearance, margination of nuclear chromatin, intracytoplasmic vacuoles, and mitochondrial swelling. In addition, the tea polyphenol-loaded chitosan NPs had relatively weak inhibitory effects on HepG2 cancer cells compared with tea polyphenols [51].

As can be seen, several studies examine the development of chitosan NPs and bioactive compounds. However, most of them are focused on the use of chitosan matrices for the controlled release of an active compound, mainly determining its rate of delivery and mostly directed to the area of pharmacology.

Essential oils, aromatic oily liquids that can be obtained from several parts of the plants, are known for their medicinal properties, mainly antimicrobial. Because these compounds can easily undergo oxidation reactions

that result in allergenic products and/or products with less biological activity, the nanoencapsulation of these oils in drug delivery systems in chitosan-based NPs formulations may be used to significantly improve the essential oil antimicrobial activity.

CONCLUDING REMARKS

The benefits and potential uses of nanotechnology are enormous. Nanotechnology enables plants to use water, pesticides, and fertilizers more efficiently. Also, their use may bring potential benefits to farmers, allowing them to innovate in the food industry via development of new products, preservation, and packaging.

Nanotechnology promises a breakthrough in improving our presently abysmal nutrient use efficiency through nanoformulation of fertilizers, breaking yield and nutritional quality barriers through bionanotechnology, surveillance, and control of pests and diseases, understanding the mechanism of host-parasite interactions at the molecular scale, development of new-generation pesticides and safe carriers, preservation and packaging of food and food additives, nanocapsules for agrochemical delivery, antimicrobial nanoemulsions for applications in decontamination of food, and improving the shelf life of vegetables and flowers, among others. Nanotechnology requires a thorough understanding of science, as well as fabrication and material technology in conjunction with knowledge of the agricultural production system. Applications in agriculture might take a few decades to move from laboratory to land.

Multidisciplinary approaches could potentially improve food production, incorporating emerging technologies and disciplines such as chemical biology integrated with nanotechnologies to tackle existing biological bottlenecks that currently limit further developments. The potential benefits of nanotechnology for agriculture, food, fisheries, and aquaculture need to be balanced against concerns for the soil, water, environment, and the occupational health of workers.

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CHAPTER 13

Biological Activity of Chitosan Nanoparticles Against Pathogenic Fungi and Bacteria

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INTRODUCTION

Nanotechnology is defined as the ability to observe, manipulate, and control the properties of matter at the nanoscale levels. Nanoscale materials (ranging from 100 nm to the size of atoms, about 0.2 nm) exhibit physical, chemical, and biological properties different from larger-size materials; also, due to their large surface area relative to the mass, nanoparticles are highly reactive. According to Foladori [1], the strength of nanotechnology lies mainly in making more efficient and multifunctional products, thereby saving raw materials. Nanotechnology has the ability to control, manipulate, and manufacture new materials with numerous applications today, reaching unimaginable limits including the ability to control individual atoms [2]. Therefore, nanoscience and nanotechnology have been major areas of scientific and technological development in the past 20 years [3].

In agriculture, nanotechnology has shown great potential in the development of major new technologies. Applications include the production and development of food-processing systems, chemicals (fertilizers, herbicides), and growth regulators. Others include the monitoring of environmental crops conditions such as humidity, temperature, and nutrition levels, the deployment of nanosensors for the detection of plant pathogens and pesticides, and the use of nanomaterials to stabilize biopesticides [2,4].

Nanotechnology also includes the study of the physical, chemical, and biological interactions between cell organelles and various diseases caused by pathogens. On the other hand, functional systems need to be designed for specific applications such as improved irrigation systems or plastic “intelligent” materials. Control of pathogenic microorganisms by applying nanotechnology is another important field of study that has been considered in this discipline.

POTENTIAL OF NANOTECHNOLOGY IN THE CONTROL OF PATHOGENIC FUNGI AND BACTERIA

Viruses, bacteria, and fungi are responsible for increased economic losses worldwide. They can cause a range of diseases, affecting different parts of cultivated plants and resulting in additional farming costs and losses. These microorganisms, over time, become more difficult to control due to the resistance generated in them by overuse of chemicals. On the other hand, to date, the chemicals used for disease control in agriculture have generated soil, river, and lake pollution, causing damage to the environment [5]. In order to provide some solutions to these problems, nanotechnology has been used. For example, the use of nanofungicides is being accepted because they are effective for plants, and depending on the nanomaterial, they cause less environmental pollution compared with conventional fungicides and bactericides [6,7].

Among other nanomaterials that have been applied in the control of plant pathogenic fungi, research conducted with silver nanoparticles and silica-silver for control of *Botrytis cinerea*, *C. gloeosporioides*, *Magnaporthe grisea*, *Pythium ultimum*, *Rhizoctonia solani*, *Sphaerotheca pannosa* var. *rosae*, *Raffaelea* sp., *Bipolaris sorokiniana*, *Fusarium semitectum*, *Fusarium solani*, *Phoma herbarum*, *Phoma glomerata*, *Alternaria solani*, *Alternaria alternata*, *Monosporascus cannonballus*, *Pythium spinosum*, *Sclerotinia sclerotiorum*, *Macrophomina phaseolina*, and *Curvularia lunata* is noteworthy. It has been demonstrated that these nanoparticles are effective in inhibiting the growth of these fungi [8–14]. However, due to the controversy that has been generated by the use of the those materials, the need to look for alternatives arises, including the use of other natural compounds such as chitosan, which has also been used in the synthesis of nanoparticles.

BIOLOGICAL ACTIVITY OF CHITOSAN IN THE CONTROL OF PHYTOPATHOGENIC FUNGI

Chitosan is a widely studied, biologically compatible biomacromolecule that is typically derived from chitin (2-acetamido-2-deoxy- β -1,4-D-glucan), the main component of the exoskeleton of insects and crustaceans, which is biodegradable and nontoxic [15]. Chitosan has a broad spectrum of activity and a significant effect in the control of phytopathogenic fungi (Table 13.1) [16–18].

Few studies have been reported for chitosan nanoparticles related to the control of plant phytopathogenic fungi. However, Kaur et al. [19], in looking for alternatives to control seed-borne pathogens in chickpea (*Cicer arietinum*), evaluated chitosan silver nanoparticles for control of *Aspergillus*

Table 13.1 Application of chitosan nanoparticles in the control of phytopathogenic fungi by different methods of nanoparticles obtention

Microorganism	Method of obtention	Control level (%)	References
<i>A. flavus</i>	Chemical reduction	94	[19]
<i>R. solani</i>	Chemical reduction	67	
<i>A. alternata</i>	Chemical reduction	78	
<i>Rhizopus</i> sp.	Ionic gelation	98	[20]
<i>C. capsici</i>	Ionic gelation	98	
<i>C. gloeosporioides</i>	Ionic gelation	98	
<i>A. niger</i>	Ionic gelation	95	[21]
<i>A. alternata</i>	Ionic gelation	82	
<i>M. phaseolina</i>	Ionic gelation	88	
<i>R. solani</i>	Ionic gelation	34	[25]
<i>A. flavus</i>	Functional group cross-linking	100	
<i>C. gloeosporioides</i>	Nanoemulsions	100	[26]

flavus, *R. solani*, and *A. alternata*, observing significant antifungal activity demonstrated by inhibition of 94, 67, and 78%, respectively. On the other hand, Chookhongkha et al. [20] evaluated the effect of chitosan on mycelial growth of *Rhizopus* sp., *C. capsici*, *C. gloeosporioides*, and *A. niger* in seeds of chili pepper (*Capsicum* sp.), showing a minimum of mycelial growth of 2.8, 2.2, 2.4, and 5.5 mm, respectively, at a concentration of 0.6% w/v. Saharan et al. [21] studied the antifungal activity of several types of nanoparticles of chitosan, chitosan with saponin and chitosan with copper, at different concentrations (0.001–0.1%). The results showed a better inhibitory effect by applying the chitosan concentration of 0.1%, which inhibited the mycelial growth of fungi such as *A. alternata*, *M. phaseolina*, and *R. solani* by 82.2, 87.6, and 34.4%, respectively. Yien et al. [22] determined the antifungal activity of chitosan in controlling *A. niger* and *F. solani*. The nanoparticles were prepared using different concentrations of chitosan of low and high molecular weight. An inhibitory effect for the low molecular weight chitosan was observed in a range of minimum concentrations of 0.86–1.2 mg/mL, while for high molecular weight chitosan, the concentrations range was 0.5–1.2 mg/mL for *F. solani*. Regarding the fungus *A. niger*, resistance to chitosan nanoparticles was observed on this microorganism. Inhibition was observed only with the nanoparticles prepared from higher concentrations of high molecular weight chitosan in a range from 1.7–2.4 mg/mL. In this study, the inhibitory effect was influenced by the particle size and zeta potential. A relationship between the size of the particle and the concentration

was also observed, being higher in those prepared with chitosan of high molecular weight compared to low molecular weight. On the other hand, Mathew et al. [23] determined the antifungal properties of chitosan nanoparticles with silver functionalized with 4(E)-2-(3-hydroxynaphthalene-2-yl) diazen-1-yl) and benzoic acid for the control of *A. flavus* and *Aspergillus terreus*. Effectiveness in the control of these fungi was demonstrated with inhibition zones of 20.2 and 27.0 mm, respectively. Brunel et al. [24] determined a minimum inhibitory concentration of chitosan nanogels for control of *Fusarium graminearum*, which was inhibited at a concentration of 5.17 mg/mL, showing that chitosan nanogels are able to interact with the cell walls of this fungus and prevent its development. Moreover, Beyki et al. [25] conducted a study on the effect against *A. flavus* using peppermint essential oil (*Mentha piperita*) encapsulated in chitosan nanogels with cinnamic acid. The *in vitro* results showed an inhibitory effect on the mycelial growth of the fungus at a concentration of 800 mg/mL. In the *in vivo* studies in tomatoes (*Solanum lycopersicon*), the results indicated the encapsulation efficiency at a concentration of 1,000 ppm for a storage period of one month at room temperature. An extension of the shelf life of the treated fruits compared to untreated ones was observed by means of higher quality of the fruit. Moreover, Zahid et al. [26] conducted an *in vitro* evaluation using chitosan nanoemulsions for controlling *C. gloeosporioides*. The results showed that the low molecular weight chitosan at a concentration of 1.0% had the best results in terms of inhibition of conidial germination of the fungus. Chowdappa et al. [27] evaluated the effect of chitosan with silver in controlling the same fungi. At concentrations of 0.1, 1.0, and 10.0 mg/mL, the growth inhibition was observed to be 44, 70, and 78%, respectively. The total inhibition of spore germination was achieved at a concentration of 100 mg/mL. *In vivo* evaluation conducted in mango fruit (*Mangifera indica*) showed that the chitosan nanoparticles with silver concentrations of 0.5% and 1% reduced anthracnose 45.7 and 71.3%, respectively. Furthermore, the combination of the chitosan with silver decreased the disease in 75.8 and 84.6% at a concentration of 0.5% and 1%, respectively.

BIOLOGICAL ACTIVITY OF CHITOSAN NANOPARTICLES IN THE CONTROL OF PHYTOPATHOGENIC BACTERIA

Agricultural products are susceptible to attack by pathogens, especially some genera of bacteria that act as agents of various diseases. However, few studies have been conducted on the biological activity of chitosan nanoparticles in

Table 13.2 *In vitro* evaluation of the effect of chitosan nanoparticles alone or amended with essential oils on *P. carotovorum* development

Treatments	Inhibition halo (cm)	CFU
Chitosan nanoparticles	$1.5 \pm 0.253b$	Countless
Chitosan nanoparticles with lime essential oil	$1.3 \pm 0.197c$	450
Chitosan nanoparticles with thyme essential oil	$2.4 \pm 0.194a$	240
Control	$0 \pm 0.0d$	Countless

Data represented as mean $\pm$ standard deviation. Different letters in the same column indicate statistical significance (Tukey, $P < 0.05$).

the control of phytopathogenic bacteria. This is the case with *Erwinia carotovora* sub. *carotovora*, recently classified as *Pectobacterium carotovorum*, which favors the disease commonly called soft rot. An *in vitro* study in which chitosan nanoparticles and chitosan nanoparticles supplemented with lime essential oil and thyme essential oil was performed. The results indicated an inhibition of the bacteria by the treatment using chitosan nanoparticles supplemented with thyme essential oil in which a halo corresponding to a value of 2.4 cm in diameter with 240 colony-forming units (CFU) was observed, while the chitosan nanoparticles supplemented with lime essential oil had an inhibition zone of 1.3 cm with 450 CFU, and chitosan nanoparticles of 1.3 cm with countless CFU (Table 13.2, unpublished data).

BIOLOGICAL ACTIVITY OF CHITOSAN NANOPARTICLES AGAINST FOODBORNE PATHOGENS

It has been demonstrated that chitosan has a biological activity against bacteria (Table 13.3). In a study performed with chitosan to control bacteria affecting human health, Shi et al. [28] achieved *in vitro* reduction of the development of *S. aureus* and *Staphylococcus epidermidis*. Specifically the number of viable bacterial cells was reduced by 103 times when in contact with this type of nanoparticles. In other studies, Ali et al. [29] synthesized chitosan nanoparticles using the ionic gelation method and tested their effect on *S. aureus*. The results indicated that particle size, concentration, and pH have an influence on the antibacterial activity. On the other hand, the inhibitory effect of the combination of chitosan with silver nanoparticles has been demonstrated in several studies. Sanpui et al. [30] synthesized silver nanoparticles with chitosan and tested their effect on *Escherichia coli*. The results showed that the bacterium was completely inhibited at a minimum

Table 13.3 Application of chitosan nanoparticles in controlling foodborne bacteria

Microorganism	Method of obtention	Control level	References
<i>S. aureus</i>	Ionic gelation	100%	[28]
<i>S. epidermidis</i>	Ionic gelation	100%	
<i>S. aureus</i>	Ionic gelation	90%	
<i>E. coli</i>	One-pot synthesis	100%	[29]
<i>E. coli</i>	Ionic gelation	100%	[30]
<i>S. aureus</i>	Ionic gelation	100%	[33]
<i>Salmonella</i> sp.	Ionic gelation	100%	
<i>K. pneumoniae</i>	Ionic gelation	100%	
<i>B. cereus</i>	Ionic gelation	100%	[34]
<i>S. mutans</i>	Ionic gelation	85%	
<i>P. aeruginosa</i>	Ionic gelation	90%	
<i>C. albicans</i>	Ionic gelation	90%	[35]
<i>E. coli</i>	Ionic gelation	100%	
<i>S. aureus</i>	Ionic gelation	100%	
<i>S. aureus</i>	Vesicle extrusion	90%	[37]
<i>L. monocytogenes</i>	Vesicle extrusion	90%	
<i>E. coli</i>	Intercalate unmodified sodium montmorillonite	15 mm	[38]
<i>Klebsiella</i> sp.	Intercalate unmodified sodium montmorillonite	15 mm	
<i>Serratia</i> sp.	Intercalate unmodified sodium montmorillonite	15 mm	
<i>Salmonella</i> sp.	Intercalate unmodified sodium montmorillonite	15 mm	
<i>Pseudomonas</i> sp.	Intercalate unmodified sodium montmorillonite	15 mm	
<i>S. aureus</i>	Intercalate unmodified sodium montmorillonite	20 mm	
<i>B. subtilis</i>	Intercalate unmodified sodium montmorillonite	20 mm	
<i>Pernela</i> sp.	Intercalate unmodified sodium montmorillonite	20 mm	
<i>M. luteus</i>	Ionotropic pre-gelation followed	100%	[39]
<i>P. aeruginosa</i>	by polycationic cross-linking	100%	
<i>S. enterica</i>	Ionotropic pre-gelation followed	100%	
<i>E. aerogenes</i>	by polycationic cross-linking	100%	[42]
<i>E. coli</i>	Cross-linking	32 mm	
<i>S. aureus</i>	Cross-linking	36 mm	
<i>B. subtilis</i>	Cross-linking	36 mm	
<i>S. faecalis</i>	Cross-linking	25 mm	
<i>P. aeruginosa</i>	Cross-linking	32 mm	
<i>N. gonorrhoeae</i>	Cross-linking	26 mm	[41]
<i>P. aeruginosa</i>	Chemical reduction method	15 mm	
<i>S. aureus</i>	Chemical reduction method	16 mm	

concentration ≥ 100 mg/mL. In a similar study, Wei et al. [31] synthesized silver nanoparticles by reducing silver nitrate salts and added chitosan, observing a significant antibacterial activity against this bacterium with a minimum inhibitory concentration of 10 mg/mL. The results also indicate that the nanoparticles of silver with chitosan have a dual mechanism of action – the bactericidal effect of silver nanoparticles and the cationic effect of chitosan – which can be useful for a wide variety of biological applications. Moreover, Vimala et al. [32] prepared chitosan films impregnated with silver nanoparticles that showed a wide inhibition in *E. coli*, *Bacillus* sp., and *Klebsiella pneumoniae* of 20, 22, and 17 mm, respectively, while Rodriguez-Arguelles et al. [33] reported a minimum inhibitory concentration of 1.3 mg/mL in *E. coli* and 2.5 mg/mL in *K. pneumoniae*, *Salmonella* sp., *S. aureus*, and *Bacillus cereus*.

Other compounds reported to increase the bacterial activity of chitosan nanoparticles include food preservatives such as nisin and erythrosine. In this regard, Chueh-Pin et al. [34] investigated the bactericidal capacity of this type of nanoparticles with erythrosine to enhance its activity against *S. mutans*, *P. aeruginosa*, and *C. albicans* by evaluating photodynamic inactivation. The results showed that bacteria exposed to darkness intervals showed no toxicity compared to those exposed to the light, where a significant decrease in viable cells was observed, demonstrating that nanoparticles combined with erythrosine improved antimicrobial effect. On the other hand, Du et al. [35] conducted studies with different metal ions such as silver nitrate, copper sulfate, zinc sulfate, manganese sulfate, and ferric sulfate, where the chitosan nanoparticles with silver ions showed a higher antibacterial activity with a minimum inhibitory concentration of 3 mg/mL and 6 mg/mL against *E. coli* and *S. aureus*, respectively. Also, Qi et al. [36] evaluated the effect of chitosan nanoparticles and copper against *E. coli*, *Salmonella choleraesuis*, *Salmonella typhimurium*, and *S. aureus*. These microorganisms were inhibited at a low concentration of 2 mg/mL for *E. coli* and *S. choleraesuis* and 4 mg/mL for *S. typhimurium* and *S. aureus*. Zohri et al. [37] also investigated the effect of chitosan nanoparticles with nisin in controlling *S. aureus* and *L. monocytogenes*. A reduction in the bacterial population after 7 days of application, with less than 106 CFU was observed. Abdeen [38] also conducted a study of chitosan nanocomposites with montmorillonite evaluating its activity against a variety of Gram-negative bacteria (*E. coli*, *Klebsiella* sp., *Serratia* sp., *Salmonella* sp., and *Pseudomonas* sp.) and Gram-positive (*S. aureus*, *Bacillus subtilis*, and *Pernela* sp.) in which the diameters of the inhibition zones were 15 mm for Gram-negative bacteria and 20 mm for Gram-positive bacteria. On the other hand, Bernela et al. [39] synthesized nisin nanoparticles with alginate chitosan and Pluronic (copolymers composed of

polyoxypropylene) for controlling *Micrococcus luteus*, *P. aeruginosa*, *Salmonella enterica*, and *Enterobacter aerogenes*. It was observed that the biological activity of nisin continued with encapsulation in polymer nanoparticles, showing an inhibitory effect up to 20 days. Rieger and Schiffman [40] incorporated cinnamaldehyde essential oil in chitosan nanofibers of poly(ethylene oxide), testing its effect on *E. coli* and *P. aeruginosa*. The results showed a high level of inactivation of both bacteria after only 30 min of contact with the nanofibers. Moreover, Lee et al. [41] evaluated the antibacterial activity of chitosan nanofibers with silver nanoparticles to control *S. aureus* and *P. aeruginosa*. Its effectiveness was proved due to the presence of inhibition zones in the range of 15–16 mm. Mohamed and Sabaa [42] synthesized carboxymethyl chitosan nanoparticles supplemented with silver. Their antibacterial activity was demonstrated against three Gram-positive bacteria (*S. aureus*, *B. subtilis*, and *Streptococcus faecalis*) and three Gram-negative ones (*E. coli*, *P. aeruginosa*, and *Neisseria gonorrhoeae*) and the fungus *C. albicans*, with high antibacterial effect. Inhibition zones were 36, 36, and 25 mm for Gram-positive, respectively; 32, 32, 26 mm for Gram-negative; and 19 mm for *C. albicans*.

CONCLUDING REMARKS

Biomolecules such as chitosan have enormous potential for antimicrobial application. By using appropriate synthesis methods, it is possible to obtain stable and highly active nanoparticles for many biological applications. In addition, chitosan nanoparticles may be a good alternative for the control of pathogenic microorganisms such as bacteria and fungi. The application of chitosan nanoparticles alone or combined with other compounds has shown their inhibitory effectiveness, which is influenced by many factors such as concentration, type of chitosan used, pH, and size of the resulting particles. Figure 13.1 shows the factors that affect antifungal and antibacterial activity against pathogens, the applications, and the advantages of using chitosan nanoparticles. Studies of these nanomaterials are mainly focused on aspects of *in vitro* control, so it is necessary to perform *in situ* control assessments to provide solutions and alternatives to the problems that the agricultural field is facing. Moreover, the modes and mechanisms of action of chitosan nanoparticles against microorganisms are not fully understood yet, thereby necessitating further research. It is also necessary to evaluate the toxic risk involved in the application of nanomaterials in the control of pathogenic microorganisms and to determine appropriate rules and measures for their use and applications.

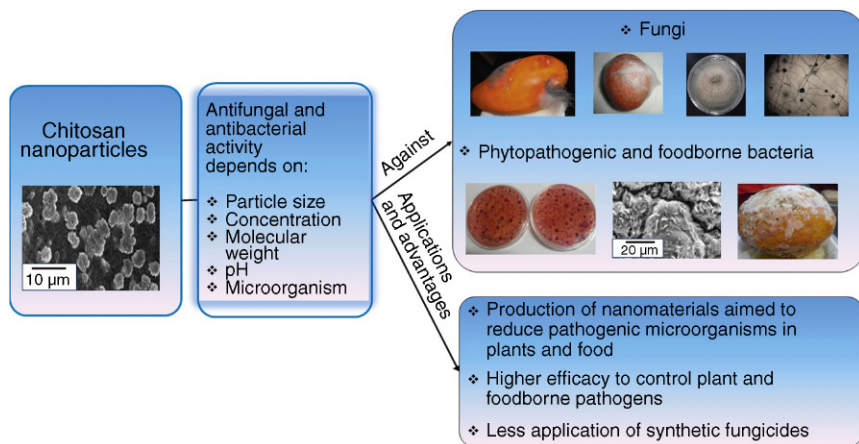


Figure 13.1 Antifungal and Antibacterial Activity of Chitosan Nanoparticles.

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CHITOSAN in the Preservation of Agricultural Commodities



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CHITOSAN in the Preservation of Agricultural Commodities presents a cohesive overview of research topics regarding the production and characterization of chitosan, the development of coatings and films, its functional properties, and antimicrobial potential of this compound on economically important agricultural commodities. It includes the modes of action from a physiological, enzymatic, and molecular perspective, and evaluations of the activity of chitosan nanocomposites and nanoparticles in biological models.

The first section deals with the chemical characteristics and functional properties of chitosan and new chitosan-based biomaterials intended for food preservation. The second section covers various aspects of the control achieved by chitosan on different microorganisms affecting various horticultural commodities, grains, and ornamentals, and its modes of action. The third section explores enzymatic and gene expression induction by chitosan application on fruit and vegetables; the fourth section offers insight on the use of chitosan nanocomposites in biological models associated with food conservation and control of microorganisms.

Key Features

- Analyzes chitosan chemical and functional properties
- Explores obtaining, characterizing, and developing chitosan coatings and films for agricultural use
- Presents functional properties, antimicrobial potential, and modes of action of chitosan from a physiological, enzymatic, and molecular perspective
- Includes biological models of the activity of chitosan nanocomposites and nanoparticles



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